

# **Elucidating the fear - maintaining properties of the Ventral Tegmental Area**

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## **Abstract**

The ventral tegmental area (VTA) and its dopaminergic (DA) mesocorticolimbic projections are thought to be essential in the brain's reward neurocircuitry. In humans and animal experimental subjects, mild electrical VTA stimulation increases dopamine levels and can induce euphoria. Paradoxically, aversive stimuli activate VTA neurons and forebrain DA activity, and excessive electrical stimulation of the VTA exaggerates fearfulness. Research suggests that experimental manipulation of either the amygdala or the VTA has similar effects on the acquisition and expression of Pavlovian conditioned fear. Recently it was demonstrated that electrical stimulation of the amygdala produced fear extinction deficits in rats. Fear extinction involves the progressive dissipation of conditioned fear responses by repeated non-reinforced exposure to a conditioned stimulus (CS). Maladaptive states of fear in fear-related anxiety disorders, such as post-traumatic stress disorders (PTSD) or specific phobias are thought to reflect fear extinction learning deficits.

The primary purpose of the present study was to examine the effects of intra-VTA stimulation on fear extinction learning. Using fear-potentiated startle as a behavioural index of conditioned fear, it was found that 120 VTA stimulations paired or unpaired with non-reinforced CS presentations impaired the extinction of conditioned fear. This effect was not apparent in rats that received electrical stimulation of the substantia nigra (SN), suggesting that not all midbrain regions respond similarly. Electrical stimulation parameters did not have aversive affects because rats failed to show fear conditioning when electrical VTA stimulation was used as the unconditioned stimulus. Also, VTA stimulation did not alter conditioned fear expression in non-extinguished animals. Based on the results it is suggested that VTA activation disinhibited conditioned fear responding. Therefore, VTA neuronal excitation by aversive stimuli may play a role in fear-related anxiety disorders thought to reflect extinction learning deficits.

# 1. Introduction

## 1.1 General introduction

The quest to determine the biology of emotions has plagued the minds of philosophers and scientists for centuries. Uncovering the biological mechanisms driving emotion is a difficult task. Not only do emotions represent transient states, but also access to a particular emotion can only be achieved by observing behaviours believed to represent that emotion. Emotions develop through interaction with the environment and these interactions are fundamentally painful or pleasurable experiences (Damasio, 2003). For example, fear is one of the most salient and readily measurable emotions that shows homology across mammalian species. Normal states of fear and anxiety are evoked by the anticipation of a negative or painful experience, typically transient, and proportional to the threat or danger encountered. The feeling of fearfulness is usually resolved by the execution of appropriate behaviours that enable the animal to deal with a fear-inducing situation, which is an adaptive mechanism promoting survival (Millan, 2003).

However, exaggerated fearfulness is intrinsically linked to the development and symptomatology of a vast array of psychiatric disturbances. The repeated experience of extreme fear is at the root of anxiety disorders ranging from specifically cued fear, such as a specific phobia or post-traumatic stress disorder (PTSD), considered a learned fear cued by intrusive memories or thoughts relating to a past traumatic experience, to a state of anticipatory fearfulness involved in panic disorder (PD) or more generalized and prolonged states of fearfulness characteristic of generalized anxiety or obsessive compulsive disorders (OCD) (Rosen & Schulkin, 1998; Foa, et al., 1995; Shuhama et al., 2007). Such anxiety disorders are described in detail in The Diagnostic and Statistical Manual of Mental Disorder IV (DSM IV, American Psychiatric Association, 1994), an internationally recognised manual referred to by many health practitioners. In addition, abnormal expressions of fear are



commonly experienced in patients with schizophrenia (Russell et al., 2007). Also, exaggerated fearfulness is a behavioural symptom accompanying temporal lobe epilepsy, and is believed to be provoked by the excessive electrical activity that induces a seizure (Gloor, 1992). Finally, hyper-excitation of fear neurocircuitry and heightened sensitivity of the stress response system has been hypothetically linked to the biological origins of childhood shyness and physical well-being (Kagan, Reznick, & Snidman, 1988; Boyce & Ellis, 2005). Thus, fearfulness can be thought of as spectrum ranging from a temporary, adaptive, and ultimately motivational state to a pathological and incapacitating condition.

Paradoxically, psychomotor stimulants such as cocaine, amphetamine, and methamphetamine, sought by substance abusers and administered to achieve a state of euphoria, can also induce temporary paranoia during the episode of drug intake, and extreme fearfulness or paranoid psychosis with chronic abuse (Griffith, Cavanaugh, Held & Oates, 1968; Bell, 1973; Robinson & Becker, 1986; Sherer, 1988; Satel, Southwick, & Gawin, 1991; Yui, Goto, Ikemoto & Ishiguro, 1997; Borowski & Kokkinidis, 1998). Paranoid psychosis can sometimes be sustained in patients withdrawing from methamphetamine as well (Sato, 1992). So, certain drugs of abuse can elicit a rewarding, subjective experience, but also extreme fear without any impending external threat. Although conceptually unconnected, it may be possible that fear and reward are biologically inter-related. Of primary interest to the author was whether excitation of a neural region activated by rewarding stimuli could also interfere with the adaptive process of relinquishing fear. Unravelling the neural mechanisms that underlie the inhibition of learned fear may be crucial to understanding the shift between adaptive to maladaptive fearfulness.

Research suggests that a singular region sub-serving fear is unlikely; instead, multiple pathways and a cascade of intra- and extra-cellular events occur to both evoke an emotion and to execute the necessary behavioural response (Damasio, 2003). One such pathway that traverses many midbrain regions, ultimately terminating in the forebrain, is the mesocorticolimbic dopaminergic pathway originating in the ventral tegmental area (VTA). The VTA has been

heavily implicated in reward learning, but more recently identified as playing a critical role in the manifestation of fear and anxiety. Converging evidence from both the reward and fear literature highlights the importance of the VTA in reinforcing motivational behaviours.

## 1.2 Anatomical characteristics of the Ventral Tegmental Area

The ventral tegmental area (VTA) is situated bilaterally in the midline on the floor of the mesencephalon. Anatomically, it is termed an 'area' because its boundaries are fairly ambiguous, bordering some major nuclei of the diencephalon (mammillary bodies and the posterior hypothalamus) and the nucleus ruber and oculomotor fibre tract. It also sits rostrally from the pons and the hindbrain (Oades and Halliday, 1987). Most importantly, fibres from many brainstem nuclei pass through the VTA forming the medial forebrain bundle (MFB), the major midbrain fibre tract that ascends toward the forebrain (Swanson, 1982; Oades and Halliday, 1987). The small collection of heterogeneous cell bodies within the VTA had been largely dismissed, historically; however, over the last two decades, the cells within the VTA have been identified as predominantly dopaminergic, and have been labelled the A10 nuclei. Moreover, due to the bi-directional connectivity between A10 nuclei and many other midbrain nuclei (Ibid, 1987), it is believed that dopaminergic pathways originating in the VTA might contribute to varying circuits in the brain that subserve the organisation, execution and regulation of motivational behaviour (Davis, 1992; Borowski & Kokkinidis, 1996; Gelowitz & Kokkinidis, 1999).

## 1.3 The VTA and reward

### 1.3.1 *Electrical brain stimulation and reward neurocircuitry*

Traditionally the VTA has been conceptualised as a key region subserving the brain's reward circuitry, therefore, governing reward-related behaviour. Reward neurocircuitry mapping was developed following Olds and Milner's (1954) initial discovery that electrical stimulation of specific cortical and

subcortical structures can be profoundly rewarding (Gardner, 2005). Particularly sensitive to the rewarding effects of electrical brain stimulation are the sites along the medial forebrain bundle (MFB) at the lateral and posterior hypothalamic and ventral tegmental levels (Wise, 1998). Evidence indicates that inter-connected neurons, which link the VTA, MFB, nucleus accumbens (Acb)- shell (DiChiara, 2002) and its closely related structures, the ventral pallidum (VP) and the medial prefrontal cortex (mPF), compose the primary reward pathways in the brain (Fiorino et al, 1993; Gardner, 1997; Wise 1998; 2000 You et al., 2001). It has been determined that either electrical or pharmacological activation of these circuits correlates with drug seeking and drug taking behaviours (Wise, 1999). Furthermore, electrical stimulation of the VTA increases dopamine transmission in rats (Fiorino, et al., 1993), suggesting that dopamine is a key neurotransmitter involved in reward.

Human studies have shown that electrical stimulation delivered to these primary reward loci can be immensely pleasurable (Gardner, 2005). Animal studies demonstrate that laboratory rats, canines, and non-human primates readily self-administer mild electrical stimulation to these same regions, even when the electrode is implanted ipsilaterally (Schwartz & Huston, 1996; Grilner & Mercuri, 2002; Gardner, 2005). Within self-stimulation paradigms, animals learn to make an association between pressing a lever and a pleasurable experience induced by electrical stimulation to a specific mesocorticolimbic region (Wise, 2002). The rewarding effects of the stimulation are behaviourally indicated by the animal's increasingly zealous lever pressing in an effort to receive further stimulations. Thus, the association between lever-pressing and reward delivery continues to be reinforced with each subsequent lever-press. Conditioned place preference methods reveal that animals exhibit a clear preference for the actual physical place/space where they received brain stimulation (Olds & Milner, 1954; Olds & Olds, 1963). And animals will even deny themselves food to the point of starvation in favour of electrical stimulation (Routtenberg and Lindy, 1965). According to early theorists Thorndike (1898; 1933), Pavlov, (1928) and Skinner (1933), rewards or reinforcers 'stamp in' learned association and response habits (In: Wise, 2002). Based on research demonstrating that stimulation of

dopaminergic regions is immensely rewarding, it could be hypothesized that dopaminergic activation may be responsible for the ‘stamping in’ of a behavioural response that is specific to a particular stimulus that cues a reward. However, although focal structures sub-serving reward neurocircuitry can be isolated via rewarding electrical brain stimulation techniques, such methods are unable to prove which neurotransmitter/s are central to the reward neural system (Wise, 1999).

### *1.3.1 Neurochemical substrates of reward system*

Direct activation of reward neurocircuitry can be achieved, however, via the administration of many pharmacological agents abused by humans for their euphorogenic properties. Drugs of abuse have highly potent rewarding effects on behaviour, which far out-weigh the pleasure derived from other biologically relevant rewards, like food, sex, and water (Gardner, 2005). The VTA has received considerable attention as a primary substrate mediating the reinforcing actions of commonly abused drugs (McBride, et al., 1999). For a brief summary of these substances see *Appendix A*. The key point is that rats will readily administer substances that up-regulate dopamine levels primarily within regions connected by the MFB (Hoebel et al., 1983; Dworkin et al., 1986; Carlezon et al., 1995; Wise, 1998). But, blocking dopaminergic transmission at VTA level, and not the Acb via local perfusion of tetrodotoxin, reduces the release of dopamine in both VTA and Acb, and is matched by a dramatic reduction, almost abolishment of self-stimulation behaviour (You et al., 2001).

How these agents excite the mesolimbic dopaminergic pathway to induce rewarding effects on behaviour, such as the enhancement of rewarding brain stimulation, is currently unclear. But it is believed that most of these agents inhibit GABAergic and glutamergic neurotransmission either pre or post-synaptically within the Acb, which might cause a dysregulation of dopaminergic excitation within the VTA (McBride, et al., 1999; Wise, 1998; Gardner, 2005; Chen et al., 2005). Nevertheless, given that approximately

70-85% of the neurons within the VTA are dopaminergic (Oades and Haliday, 1987), the importance of the aforementioned reward research to the current study is that either electrical or pharmacological activation of dopamine neurons in the VTA is crucial to motivational behaviour. Also relevant is that the rewards of animal self-administration studies are unsensed- without a specific smell, taste, sound, texture, visual marker; either electrical stimulation or psychopharmacological agents are delivered directly to discrete areas in the brain, for example the VTA, triggering a subjective experience of pleasure that is anatomically specific (Wise, 2002). Thus, the context surrounding reward delivery and the behaviour required to recreate a pleasurable sensation (e.g. bar-pressing) must become critically important factors in creating the rewarding experience, which is repeatedly reinforced with subsequent stimulations. This observation is aptly described by E.L. Thorndike in 1911: "Any act which in a given situation produces satisfaction becomes associated with that situation so that when the situation recurs that act is more likely than before to recur also. (In: Redgrave, Prescott & Gurney, 1999).

### *1.3.2 Conflicting views: The role of dopamine in reward governed behaviours*

There is little agreement between researchers as to the specific action of dopaminergic neuromodulators in reward learning, particularly given that dopamine functions in a diverse range of processes from movement to drug addiction (Chinta & Anderson, 2005). The most established argument is that dopamine drives feelings of pleasure, which is supported by findings that dopamine levels increase in response to drug self-administration. The claim is that the primary function of dopaminergic neurons is to encode hedonic tone (Wise, 1985; Koob & Le Moal 1997; Gardner, 2005), meaning that the release of dopamine is required for a pleasurable or 'liking' sensation. But, reward driven behaviour has a variety of cognitive components that are difficult to tease apart. For example, how do we differentiate between desire, pleasure, and a habitual when considering patterns of addictive behaviour? However, recently Robinson et al., 2005, demonstrated that dopamine function is necessary to generate only the 'wanting' aspect of reward oriented behaviour,

as opposed to liking or learning about rewards (Berridge, 2005). Such ingenious studies help to refine our understanding the role of dopamine neurons in maintaining reward driven behaviour.

An alternative theory suggests that dopamine neurons encode prediction errors associated with rewards (Schultz, 1998). In support of this view, primate studies have demonstrated that midbrain dopamine neurons are reliably activated when rewards are unexpected (Hollerman & Schultz, 1998). When reward delivery is signalled by a neutral cue, dopaminergic firing is activated by the predictive cue, rather than the reward itself (Hollerman et al., 1998). Also, dopaminergic firing will begin to diminish if the reward is repeatedly withheld, suggesting that dopamine neurons can detect deviations from expected reward (Schultz, 1998; Wise, 2002).

According to this view prediction errors are recorded within the VTA and the substantia nigra pars compacta (SNc), but these two dopaminergic areas perform different, yet intimately related processes (Dayan & Balleine, 2002). Thus, VTA derived dopaminergic pathways to the basolateral nucleus of the amygdala and the orbito-frontal cortex govern value learning and/or incentive processes (Alexander and Crutcher, 1990; Suri & Schultz, 1999; Wise, 2002; Dayan & Balleine, 2002). And, the SNc controls action (Alexander and Crutcher, 1990; Dayan & Balleine, 2002), enabling a motor response (Hornykiewicz, 1979; Carlson, 2001). The Acb is thought to be the interface between these two systems, which marries a value-based prediction with an adaptive choice of action (Suri & Schultz, 1999; Wise, 2002). Simultaneous activation of dopaminergic pathways is believed to play a pivotal role in reinforcing the association between reward cues and an appropriate motor response. This is also supported by electrophysiological studies that demonstrated changes in burst firing of dopamine neurons of the SN correspond with behavioural changes made during reward learning tasks (Hyland et al., 2002). Additionally, dopamine has been shown to regulate striatal synaptic plasticity, suggesting that dopamine neurons are involved in the modulation of long-term potentiation (LTP), which is presently the best

molecular model of associative learning processes (Calabresi et al., 1992; Reynolds & Wickens, 2002).

Conversely, Redgrave et al., (1999) have proposed that dopamine neurons play an important role in shifting attention and behavioural resources toward unexpected, salient events. They demonstrated a change in firing activity of dopamine neurons in response to unexpected salient events involving stimuli that are novel, sudden, and intense. The stimuli must be, or predict something that is, biologically beneficial to the animal (Ibid, 1999). However, 'survival' is definitely of biological benefit, and therefore, dopamine neurons must also respond to stimuli that are threatening or fear provoking. Horvitz (2000) also asserts that dopamine neurons respond to salient events encompassing all circumstances in which environmental conditions change, which includes non-rewarding events/stimuli. Microdialysis evidence indicates mesolimbic dopaminergic increases during the presentation of aversive stimuli and prior to stimuli offset (Puglisi Allegra et al., 1991; Horvitz, 2000). Because it was demonstrated that the dopamine response is immediate, rather than triggered during aversive stimulus offset, it could not be explained as a neuronal reaction to a rewarding event - the reward being the cessation of an unpleasant experience.

In sum, there is controversy surrounding the view that dopamine neurons respond to aversive stimuli and not just rewarding stimuli. But, the evidence is conflicting probably because experimental methods vary considerably. The three basic views concerning the main role of dopamine neurons originating in the midbrain are: 1) dopamine neurons underlie the subjective feeling of pleasure; 2) dopamine neurons enable the prediction of reward delivery, which suggests involvement in associative learning processes; or 3) dopamine neurons respond to all salient stimuli, which may include aversive stimuli. If these dominant views are combined it can be theorized that dopaminergic activation is necessary for motivational behaviour, traditionally conceptualized as positive, but could also include reactions to negative events or a more generalized 'shift' in behaviour to attend to any new and biologically relevant

stimuli. Therefore, investigations into dopaminergic functioning must consider alternatives to solely reward related paradigms.

## 1.4 Stress and the mesolimbic system

### *1.4.1 The dopaminergic system mediates both reward and stress*

Research indicates that the mesolimbic dopaminergic system is also stress responsive, and contributes to the mechanisms that mediate the manifestation of fear and arousal (Millan, 2003).

Dysregulation of dopamine neurons in response to stressful stimuli is believed to play a role in the manifestation of schizophrenic symptomatology (Finlay & Zigmond, 1997). Moreover, negative affective states including dysphoria and anxiety that can accompany schizophrenia are also common symptoms arising from amphetamine withdrawal (Koob & Le Moal, 1997; Pezze & Feldon, 2004). Additional evidence indicates that former addicts are more likely to relapse in response to stressful circumstances (Piazza & Le Moal, 1998). Animal studies have also shown that rats previously exposed to psychomotor stimulants exhibit a greater sensitivity to stress (Kokkinidis, 1983; 1984).

Koob and Le Moal (1997) hypothesized that dysphoria associated with drug withdrawal is a dual product of decreased VTA – MFB - Acb reward function combined with an increase of the stress induced neurotransmitter corticotrophin releasing factor (CRF) in the central nucleus of the amygdala. The amygdala is commonly associated with the manifestation of learned and unlearned fear, and neuronal plasticity relating to long-term-potential is believed to underlie learning processes is also evident in the amygdala (LeDoux, 2000). Recently, Saal et al., (2003.) demonstrated that both typically abused drugs and exposure to acute stress independently increased strength at excitatory synapses on dopamine neurons promoting neuronal plasticity in the VTA. However, this plasticity was absent when RU486, an



antagonist of glucocorticoids, was administered prior to either experimental manipulation. (Glucocorticoids are hormones activated during times of stress). Therefore, irrespective of whether heightened stress levels are the cause or effect of elevation of dopamine levels in the brain, the evidence indicates that the reward and stress response systems are inter-related and mediated by dopamine neurons.

#### *1.4.2 Dopamine neurons and emotional responses to aversive stimuli*

Despite the evidence linking dopaminergic VTA neurons to the stress response system, the precise actions of these neurons in response to aversive events is unclear. When responding to sudden, stress-inducing stimuli, dopamine neurons within the VTA trigger dopamine release primarily in the Acb (Tidey and Miczek 1996; Imperto et al., 1992) and PFC (Sorg and Kalivas 1993), a response akin to the reaction of mesolimbic dopamine neurons to rewarding stimuli (Wise, 2002). Novel or moderately unpleasant stimuli such as tail-pinch or psychosocial stressors also have been shown to increase (in vivo) mesolimbic DA release (Doherty and Gratton, 1996; Tidey and Miczek, 1996). Other studies suggest that elevations in dopamine activity occur in response to only very aversive stimuli (Salamone et al., 1997) like footshock (Sorg & Kalivas, 1991), tail-shock (Keefe, et al., 1993), tail-pinch (Kiyatkin, 1988), extreme cold exposure (Keller, et al., 1983), and restraint stress (Doherty et al., 1997). Whether midbrain dopaminergic neurons respond to mild versus acute and sudden stressors remains unclear due to conflicting research findings.

Of particular relevance to this study is whether VTA-derived dopamine neurons are activated in anticipation of an aversive event, which enables a learned fear response. Trulson & Preussler (1984) recorded enhanced activity of VTA dopamine neurons in cats responding to a tone that had been paired with a moderately aversive air-puff to the eye. Additionally, extra-cellular recordings taken from the VTA in rabbits during Pavlovian conditioning

demonstrated that the firing rates of dopamine neurons in the VTA change in response to stimuli that predicts an aversive event (Guarraci & Kapp, 1999). But, the finding that dopamine depleted rats were able to translate a Pavlovian association between sweet and aversive tastes (Berridge and Robinson, 1998) suggests that not all forms of Pavlovian conditioning rely on dopamine input. Rather, the evidence seems to suggest that dopamine neurons become involved when the organism is under stress and increased dopamine release represents a generalized state of arousal in the animal, which encompasses both positive and negative emotional states (Ikemoto and Panksepp, 1999). Additionally, it has been argued that the level of increase is dependent on the intensity of environmental changes (Imperato, et al., 1992).

#### *1.4.3 Possible functional dissociation between mesolimbic and nigrostriatal dopamine neurons*

The understanding that all midbrain dopaminergic neurons act in the same manner is also controversial. Horvitz (2000) has argued that VTA and SN dopamine neurons are similarly activated by a broad range of sensory stimuli. In support of this, electrophysiological studies have shown single dopamine cells of both the VTA (Kiyatkin, 1988; Horvitz, et al., 1997) and the SN (Chiodo, et al., 1980) exhibit an alteration in firing rates in response to aversive stimuli. In contrast, a microdialysis study demonstrated that mild stress or exposure to a fear-evoking stimulus increased dopamine metabolism in the VTA indicating dopaminergic neuronal activation, which was not observed in the SN (Deutch et al., 1985). Despite this regional difference, as yet, there is not sufficient evidence to support a functional dissociation between nigrostriatal and mesolimbic dopaminergic systems. Furthermore, conflicting views regarding dopaminergic responses to differing stimulus input are difficult to resolve because different studies use different methods to measure dopaminergic functioning. For example, the measurement of extra-cellular dopaminergic concentrations within specific regions produces different findings than the recording of individual action-potentials within a single cell, and increased firing activity of a dopamine neuron does not necessarily result

in an enhancement of dopamine release (Horvitz, 2000). Neurobehavioral studies can connect observable behaviours to the actions of dopamine, which can also be practical evidence for previous cellular studies and theories that are more abstract.

In summation, mesocorticolimbic and nigrostriatal dopamine neurons respond to a vast array of arousing events suggesting that dopaminergic neurons mediate a number of behavioural functions. Based on the evidence gathered from the reward and stress literature, it is evident that mesolimbic dopaminergic neurons respond to both external and internal (physiological) environments, which can be either rewarding or aversive. Whether dopamine neurons are more responsive to emotionally arousing stimuli in mesolimbic regions compared to dopaminergic neurons within nigrostriatal regions has yet to be determined. Certainly there are legitimate reasons to explore the role of the VTA in the generation of emotional states, such as fear

## 1.5 Fear and the mesolimbic system

### 1.5.1 *The behavioural response to fear*

Fear is a normal emotional response to threatening stimuli, which can be accompanied by both endocrine and various autonomic physiological responses. These anatomical responses trigger the fight or flight response that enables an animal to respond appropriately to impending danger. Fearfulness is not only experienced subjectively, it is one of the few emotional states that is also recognized as a distinct set of observable behaviours governed by a complex neural network (Pezze & Feldon, 2004). For all animals, learning the predictive relationship between aversive and environmental stimuli is a necessary, protective mechanism that ultimately promotes survival.

### *1.5.2 Neural mechanisms underlying normal fear*

Behavioural expressions of fear involve complex process. These are thought to occur, and be modulated within, common mesocorticolimbic structures such as the amygdala, hippocampus, and the cortex (Millan, 2003; LeDoux, 2000). It is largely believed that primary emotional and motivational functions are initially formed sub-cortically within limbic structures and interconnected descending pathways (Morgane, Galler, and Mokler, 2005). Because dopaminergic secretion is believed to reinforce behavioural responses (Carlson, 2001), and dopamine neurons are thought to respond to aversive stimuli (Saal et al., 2003; Horvitz, 2000; Doherty et al., 1997; Salamone et al., 1997; Doherty and Gratton, 1996 ; Tidey and Miczek, 1996; Sorg and Kalivas, 1991; Keefe, et al., 1993; Deutch et al., 1985; Keller, et al., 1983; Kiyatkin, 1988), mesolimbic dopaminergic pathways originating from the VTA are thought to play a critical role in learned fear (Millian, 2003; Morgane, Galler, and Mokler, 2005).

### *1.5.3 Abnormal fear: electrical stimulation of the amygdala and VTA evokes exaggerated fearfulness*

In the case of abnormal fear, exaggerated fearfulness results from direct electrical stimulation to the human amygdala (Gloor, 1992; Grillon, 2002). Similarly, inter-ictal behavioural disturbances, thought to be caused by abnormal electrical activity in temporal lobe epileptic patients, often involve irrational and intense fearfulness (Klaynchuk, 2000; Depeulis et al., 1997). It is assumed that such electrical stimulation produces hyper-excitability in fear circuits, mediated by the amygdala and the bed nucleus stria terminalis (Rosen and Schulkin, 1998), with the latter region governing non-specific fear (Walker et al., 2003).

Manipulation of dopamine neurons within the VTA can also alter the expression of fearfulness. Since Stevens and Livermore (1978) discovered that high frequency electrical stimulation of the VTA elicited exaggerated fear

responding in cats, there has been mounting evidence to suggest that mesoamygdaloid dopaminergic transmission originating in the VTA may be necessary for fear arousal (Millan, 2003, Nader and LeDoux, 1999; Horvitz, 2000; Gifkins et al., 2002). Conversely, ablation of the VTA produces hypo-emotionality in rats, presumably due to elimination of dopaminergic transmission at the original locus of the mesolimbic system (Le Moal et al., 1969). In further support of the possible inter-dependent relationship between the VTA and the amygdala, repeated sub-convulsive electrical stimulation of the VTA has been shown to induce neural sensitization within the amygdala, which could not be explained as an electrophysiological artefact of after-discharge threshold alteration (Gelowitz and Kokkinidis, 1999). Additionally, electrical or pharmacological excitation of dopaminergic neurons in the VTA enhances the acoustic startle reflex; whereas, excitotoxic lesions and intra-VTA infusion of D<sub>2/3</sub> receptor agonist block fear potentiated startle (Gelowitz and Kokkinidis, 1999; Borowski, and Kokkinidis, 1996; 1998). These findings suggest that neural hyper-excitation within the amygdala, evoked either organically or artificially, could be modulated by dopamine neurons within the VTA.

#### *1.5.4 Conditioned fear*

In a laboratory setting, Pavlovian fear conditioning is a key experimental paradigm that demonstrates the most basic and primitive form of associative learning. During Pavlovian conditioning, an organism learns that a specific innocuous stimulus (conditioned stimulus [CS]), such as a tone or a light, predicts an aversive consequence or event (unconditioned stimulus [US]) like shock (Pavlov, 1927). After only a brief exposure to the CS + US pairings, laboratory animals display behaviours remarkably similar to human symptoms of fear and anxiety, such as: hyper-vigilance, anxious anticipation and avoidance, in response to the conditioned stimulus alone (Maren, 2001). Therefore, cued fear conditioning involves a learned fear response that is associated with an explicit cue signalling danger, thus eliciting freezing behaviour and/or increased startle (Grillon, 2002), which in the absence of the

US is deemed a conditioned response. Importantly, over-excitation or inhibition of dopamine transmission via administration of dopamine agonists or antagonists enhances or attenuates conditioned fear responding, respectively (Pezze and Feldon, 2004).

### *1.5.5 Fear potentiated startle*

In the laboratory in the current study was conducted, fear potentiated startle (FPS) is preferred as a behavioural index of conditioned fear. This method recognises that during states of fear, the startle reflex is reliably evoked when an animal or human anticipates an aversive event / stimulus (Davis, 1992). Typically, acoustic startle amplitude is used as an index of fearfulness in animals, whereas eye-blink startle is more commonly used in humans (Grillon, 2002).

To determine whether rats have successfully learned the association between the CS and the US, on the test day following the Pavlovian conditioning procedure, they are presented with a series of noise bursts at decibel levels of between 90-105 db, which produces dependable startle in the animals. Baseline startle amplitudes are compared to the startle amplitudes rats' exhibit when the acoustic startle stimulus is paired with the CS. The difference in startle amplitudes between the two stimuli conditions provides a quantifiable and standardized measure of fear that can be compared across animals of different experimental conditions (Davis, 1992).

FPS is a form of Pavlovian conditioning and is reliably and easily induced in both animals and humans producing similarly consistent effects on behaviour. Hence, FPS is favoured for its high face validity. In humans, fear potentiated startle is augmented in the presence of a neutral stimulus conditioned with an electric wrist shock (3.0 mA, 10-s duration) (Grillon, et al., 1991; Chan and Lovibond, 1996). It has also been found that unpredictable delivery of the US (an unawareness of the CS-US association) increased anxious and avoidant

behaviours (Grillon, 2002). Successful fear conditioning was achieved using virtual reality methods (Pine et al., 2001). It has also been demonstrated that cue specific fear associations can be maintained, despite dramatic environmental changes surrounding the conditioned cues (Baas, et al., 2004). This would suggest that the memory of the explicit CS-US association is extremely strong in both animal and human, a learned association that generalizes across different contexts. Finally, because fear and anxiety augment startle responses, fear potentiated startle has become a suitable vehicle to investigate conditioned fear.

### *1.5.6 The amygdala and Pavlovian conditioned fear*

The neural mechanisms underlying fear conditioning, most prominently within the amygdala, appears homologous across several mammalian species as amygdala ablation in laboratory animals and amygdala pathology or amygdectomy in humans result in Pavlovian fear conditioning deficits (Blanchard and Blanchard, 1972; Bechara et al., 1995; Buchel et al., 1999). The severity of impairment is typically proportional to the extent of amygdala damage (e.g. bilateral vs. unilateral) (Maren, 2001).

A tremendous body of animal research has clarified the role of the amygdala by delineating the neural circuits underlying Pavlovian fear conditioning. Anatomical and behavioural evidence suggests that there are two functionally distinct sub-systems within the amygdala that are important to fear conditioning (LeDoux, 2000). Basolateral and lateral amygdaloid nuclei (BLA) support and integrate fear sensory inputs, primarily via the thalamus; information about the conditioned stimulus is then projected to central amygdaloid nuclei (Davis, 1992; LeDoux, 2000). Thus, the BLA is thought essential to the acquisition and expression of conditioned fear.

The central amygdala (CE) and its reciprocal connection with the periaqueductal gray (PAG) mediate behavioural, autonomic and endocrine responses, which constitute learned fear responses (LeDoux, 2000; Parades

et al., 2000; Millian, 2003). In particular, the ventral amygdalofugal (VAF) pathway between the CE amygdala and the caudal pontine nucleus of the reticular formation (PnC) mediates fear potentiated startle (Fendt and Fanselow, 1999). Although lesions of BLA and CE amygdaloid complex suppress the acquisition and expression of fear learning (Davis, 1992; Maren, 2001; LeDoux, 2000), lesions specific to the CE are presumed to cause fear response impairments rather than deficits in forming conditioned fear associations (Fanselow and Kim, 1994).

#### *1.5.7 The role of the VTA in Pavlovian conditioned fear*

Although extensive investigations have differentiated the roles of amygdaloid nuclei in the acquisition, manifestation, and maintenance of learned fear, there is emerging evidence that lower midbrain structures, particularly the VTA, are also critically involved.

The VTA is thought to contribute to the neurogenesis of fear arousal and learning via ascending projections to sub-amygdaloid nuclei (Oades and Haliday, 1987; Munroe and Kokkinidis, 1997; Millian, 2003; Nader and LeDoux, 1999; Horvitz, 2000; Giftkins et. al., 2002). A high volume of dopamine is found in the central amygdala (CE) and its reciprocal connections to the peri-aqueductal grey (PAG). The PAG is deemed vital to autonomic, endocrine and behavioural fear responses (Millian, 2003). Dense dopaminergic fibre clusters innervate the basolateral amygdala (BLA) (essential to the integration of fear sensory information received primarily from the thalamus (LeDoux, 2000; Maren, 2001)), and are believed to be A10 DA neurons (Oades and Haliday, 1987) deriving from the VTA. Dopaminergic terminals are located in the amygdala, Acb, and the mPFC. These are key regions driving conditioned fear, and target structures for the mesolimbic dopaminergic system originating in the VTA (Pezze and Feldon, 2004). Blocking dopamine D<sub>1</sub> receptors peripherally (Davis et al., 1993) and directly within the VTA (Greba and Kokkinidis, 2000) blocks fear potentiated startle.



Delivery of dopamine D<sub>2</sub> agonist quinpirole stimulates D<sub>2</sub> auto-receptors, thus reducing dopamine production and release, and as a consequence, attenuates conditioned fear responding (Nader and LeDoux, 1999).

Because the blockade of dopamine transmission from the VTA into the basolateral amygdala inhibits fear responding, it is possible that dopaminergic output from the VTA evoked by a stressful event could represent the neurogenesis of learned fear. Hence, in rats, electrical stimulation of neurons within mesolimbic sites, particularly the VTA, can exacerbate startle or freezing responses to conditioned stimuli (Rosen et al., 1996; Kalynchuk, 2000). Therefore, it is also probable that dopaminergic hyper-excitation originating in the VTA maintains heightened fearfulness and overrides the adaptive ability to terminate a fear response in a non-threatening environment.

## 1.6 The inhibition of fear: extinction learning

### 1.6.1 *Clinical implications of extinction learning deficits*

Fear provoking associations are rapidly learned and long remembered, making the relinquishment of learned fear very difficult; and yet, the ability to inhibit overt fearfulness is a necessary and adaptive function of human behaviour. In fact, it was recently theorized that patients presenting with anxiety disorders like PTSD, agoraphobia, or specific phobias, may possess a generalized inability to inhibit abnormal fear responding across non-threatening contexts (Cain et al., 2003; Jovanovic et al., 2005). Clinically, exposure therapy, or flooding as it is sometimes known, has been the most successful treatment for specific fear related psychological disorders (Garakani, et al., 2005). Based on a simple behavioural technique known as fear extinction, its aim is to disrupt the association between the CS and the US by exposing the subject to repeated presentations of the CS in the absence of the US (Pavlov, 1927). Importantly, extinction learning does not eliminate previous fear associations or enable the patient to forget them (Myers and Davis, 2002). Rather, it is the dominant view that extinction of conditioned

responses represents the formation of a new association linked to the CS; which masks the first excitatory fear association between the CS and US (Bouton, 2002; Millan, 2003). Therefore, it is possible that fear related psychopathologies could arise from fear extinction learning deficits. But, the precise neural abnormalities that retard extinction learning processes, remains uncertain.

### *1.6.2 The animal model of extinction*

The extinction paradigm has become a popular experimental technique adapted by neurobehaviourists to model inhibitory fear learning (Bouton and Bolles, 1979; Bouton, 2002). Following Pavlovian conditioning (typically using footshock) and subsequent fear testing, animals undergo extinction training. In normal animals, on the post-extinction fear potentiated startle test, there is a reliable reduction in frequency and amplitude of fear responses to the CS that previously predicted footshock. This indicates successful extinction of conditioned fear.

Recently, Myers and Davis (2002) distinguished between within session extinction and extinction retention. The former relates to a reduction in conditioned responding during extinction training, and the latter relates to the absence of conditioned responding after the extinction training session (usually after 24 hours). This distinction is particularly important. A within session impairment suggests an acquisition and/or expression of an extinction learning deficit; but retention means that consolidation or retrieval extinction memory processes could be compromised. Although the two extinction conditions are not necessarily mutually exclusive in terms of a resultant behavioural deficit, this procedural difference becomes vital when attempting to assign neural regions to particular extinction learning processes. Hence, temporary neural alterations induced by transient manipulations and delivered at specific times during extinction training are perhaps more enlightening than permanent ablations of particular neural regions prior to training. This important consideration underscores the benefits of using localized electrical

or pharmacological stimulation rather than lesions on specific brain regions for an animal model of extinction learning.

### *1.6.3 The importance of context to extinction learning*

It was initially demonstrated by Bouton and Bolles (1979) that following extinction the CS acquires a second, neutral meaning that co-exists with the initial conditioned CS + US association, which signals fear. This dual association is evident because conditioned fear responses can be reinstated following successful extinction if the subject is re-exposed to the US. So, the initial CS-US association is not forgotten (Bouton et al., 2005).

However, extinction learning appears to be entirely dependent on the context of retrieval (Bouton, 2002). The phenomenon of conditioned fear reinstatement after extinction is context dependent because if the US is delivered in an irrelevant context, the reinstatement of conditioned fear is not observed (Bouton et al., 2006). Conversely, conditioned fear is renewed if the subject is tested in an environment that differs from the extinction training and testing context. In addition, not only is extinguished conditioned fear highly sensitive to changes in external (background stimuli, temporal factors) contextual cues, but internal (drug/mood state) contextual cues also contribute to the instability of fear extinction (Bouton & Bolles, 1979). For example, in one study, rats received extinction training while under the influence of benzodiazepine tranquilizers, but when these animals were tested without the influence of drugs, conditioned fear was renewed (Overton, 1985). Additionally, in humans, a memory can be difficult to retrieve if the subject is not in the same mood or emotional state as when the memory representation was formed (Eichenbaum, 1995). Evidence of spontaneous fear recovery, when a large time-lag between extinction training and subsequent testing exists without any non-reinforced CS exposure, indicates that the temporal context is also critical (Bouton, et al., 2006).

Thus, consideration of time, space and emotional state are significant factors

contributing to the success and maintenance of extinguished fear. Moreover, the existence of reinstatement, renewal, and spontaneous recovery exemplifies the fragility of extinction, and because maintaining extinguished fear in animals is difficult, this suggests that the new inhibitory association requires reinforcement if it is to be sustained (Myers & Davis, 2002).

By contrast, Pavlovian fear conditioning is considerably less influenced by context (Bouton, 2002), and as a consequence, is a more robust learned association. Based on this observation Bouton (2002) postulated that differences in context dependence between conditioned fear learning and extinction learning could underlie the pervasiveness of fear related behavioural disorders. However, despite evidence of conditioned fear renewal and reinstatement (which are context dependent) after completion of extinction training, conditioned responding is of less magnitude in animals that have never undergone extinction training (Bouton, et al., 2005). This suggests that some element of extinction learning is not associated with context, and that contextual cues modulate rather than dictate fear extinction (Ibid, 2005).

## 1.7 Proposed anatomical explanations for extinction

### *1.7.1 Medial prefrontal cortex: consolidation and retention of extinction memory*

In an effort to locate the neural mechanisms underpinning extinction learning, neuroscientists looked first to the prefrontal cortex (PFC). This region has been repeatedly implicated in controlling behavioural inhibition, which is a primary component of adaptive cognitive skills (Myers & Davis, 2002). However, involvement of the PFC in the extinction of conditioned fear is ambiguous with many lesion studies reporting conflicting findings. For example, Morgan et al., (1993) reported impaired extinction of conditioned responding to tone, but not context extinction retardation following vmPFC lesions. Quirk et al., 2000, also reported extinction retention, but not within-session deficits when the vmPFC was lesioned. Conversely, no effect on extinction learning was reported following dmPFC (Morgan & LeDoux, 1995)

and vmPFC (Gerwitz et al., 1997; Morgan & LeDoux, 1999) lesions. It has been argued that the medial PFC is the critical site subserving consolidation and retention of extinction memory, rather than extinction acquisition (Quirk et al., 2000; Santini et al., 2001). But, monoaminergic transmission within the mPFC, disabled using 6-OHDA, has been shown to leave rats unable to acquire the new extinction association within the training session (Morrow et al., 1999). However, Milad and Quirk (2002) have demonstrated an extinction learning enhancement in rats that receive electrical stimulation of the cortex during extinction training, which suggests that PFC activation contributes to extinction acquisition.

From a clinical perspective, it is necessary to isolate key structures underlying fear extinction learning, particularly if internal arousal states (drug or mood induced) might interfere or enhance the efficacy of extinction-based treatments (Bouton, 2002; Garakani, 2006). Given the conflicting reports on the mPFC and extinction learning, and the fact that that extinction acquisition and retention might involve two entirely separate structures, it is more likely that the mPFC acts in concert with other neural regions and inter-connected pathways supporting fear arousal and suppression.

### *1.7.2 Hippocampal involvement: context dependent extinction memory*

Based on the well established belief that the hippocampus is an integral region subserving contextual aspects of memory; i.e. the encoding of time and space (Squire, 1992; Eichenbaum and Cohen, 1993; Kesner, 1998), there is a high probability that the hippocampus is involved in processing the contextual elements of extinction learning (Bouton et al., 2006). In particular, the hippocampus has been shown to support contextual aspects of Pavlovian conditioned fear (Fanselow, 2000). Animal studies have shown that hippocampal lesions, sometimes including ablation of the fimbria-fornix, impair the renewal and reinstatement of previously extinguished conditioned fear (Corcoran and Maren, 2001; Ji and Maren, 2005; Frohardt et al., 2000).

Temporary inactivation of the dorsal hippocampus by administering muscimol, a GABA receptor agonist, before retention testing, impaired the retrieval of contextual components of extinction memory (Corcoran et al., 2005). However, as yet no study has demonstrated conclusively a direct association between a complete extinction deficit and hippocampal inactivation (Bouton, et al., 2006; Kim and Fanselow, 1992). But, there have been findings that suggest hippocampal lesions, before conditioned appetitive extinction training, retard the rate of extinction acquisition (Benoit et al., 1999). The attribution of the hippocampus to all contextual elements of extinction learning and memory is problematic because of a reasonable possibility that different regions support the processing, encoding, integration, and storage of contextual-extinction information. Thus, hippocampal integrity may be critical for the retrieval of context-dependent aspects of extinction memory, possibly explaining why renewal and reinstatement deficits have been observed after hippocampal lesions (Corcoran and Maren, 2001; Ji and Maren, 2005; Frohardt et al., 2000). However, the acquisition and formation of contextual associations directly related to the acquisition of extinction learning, like mood states or emotional arousal, could be mediated by mesolimbic regions more critically related to learned fear, such as the amygdala (Davis, 1992; Rogan and LeDoux, 1996).

### *1.7.3 Mesoamygdaloid involvement: neurobehavioural evidence*

Based on the compelling evidence that amygdaloid dopaminergic transmission mediates the acquisition and expression of conditioned fear (Borowski & Kokkinidis, 1996; Greba & Kokkinidis, 2000), and that neural plasticity within the amygdala is evident once CS-US conditioned associations are learned (LeDoux, 2000), researchers begun to investigate amygdaloid involvement in fear extinction. The amygdala is also believed to mediate conditioning to context via its connection with the hippocampus (Myers & Davis, 2004), which in conjunction with the lateral septum, are thought to process contextual elements (an important aspect of extinction associations) of extinction learning (Schmajuk, 1984).

Recently Kellett and Kokkinidis (2004) found that intra-amygdala electrical stimulations, paired with non-reinforced CS presentations, impaired the extinction of Pavlovian conditioned fear in rats, in comparison to control animals that received only non-reinforced CS presentations (normal extinction training). All animals received a fear potentiated startle test 24 hours after extinction training using 120 non-reinforced CS presentations, which means that Kellett and Kokkinidis were testing for a retention (delayed test) rather than an acquisition (within session) extinction deficit. It could be reasoned that the amygdaloid stimulation used in their experiment, disturbed the reduction of neuronal firing that normally occurs when the presentation of the CS is not explicitly paired with a US (Collins and Pare, 2000). The authors also demonstrated that amygdaloid stimulation after extinction learning reinstated initial conditioned fear responding (Kellett & Kokkinidis, 2004).

A similar reinstatement of extinguished conditioned fear responding was observed in an earlier study conducted by Bouton, (1984) when he re-exposed laboratory animals to a brief set of footshocks. Kellett and Kokkinidis (2004) showed that stimulation-evoked fear reinstatement was context specific, as conditioned responses did not return when animals received amygdaloid stimulations in a chamber distinct from the extinction training and testing context (Kellett & Kokkinidis, 2004). Fear potentiated startle was not produced when amygdala stimulation was used as the US, which suggested that the provocation of unconditioned fear by the stimulation was not a sufficient explanation for these findings (Ibid, 2004). Finally, there was no evidence that 120, 1 second electrical stimulations of 30  $\mu$ A reduced after-discharge threshold levels, which is the first indication of seizure induction indicated as augmented neural activity on an electroencephalogram (EEG), (Goddard, et al., 1969; Racine 1972). Findings from this study demonstrate that electrically evoked amygdala excitation produced effects that were specific to conditioned fear extinction learning.

## 1.8 Possible neuronal processes underlying extinction

### 1.8.1 *Dopamine and extinction*

Dopamine (DA) neurons are under tonic GABA inhibitory influence (Paladini et al., 1999; Sesack & Carrb, 2002) and some researchers believe that GABA neurons mediate the expression and retention of extinction learning (Harris & Westbrook, 1998). Dopamine neurons are also believed to play a role in extinction learning because DA has been implicated in motivation and motor learning (Koob, 1996; Redgrave et al., 1999; Reynolds & Wickens, 2000). Borowski and Kokkinidis (1998) discovered that non-contingent systemic administration of specific D1 receptor agonist SKF 38393 provoked the restoration of a CS cued fear response in previously fear-extinguished rats. Similarly, systemic administration of D2 receptor agonist quinpirole prior to extinction training also impaired extinction acquisition (Nader & LeDoux, 1999). Therefore, it would be reasonable to postulate that these extinction deficits were produced by dopaminergic provocation of an enhanced fear response. Overall, the neuroanatomical evidence suggests that mesolimbic dopaminergic pathways are intrinsically related to both the inhibition and disinhibition of Pavlovian conditioned fear.

## 1.9 Hypothesis: Electrical stimulation of the Ventral Tegmental Area during extinction training creates an extinction learning deficit.

Research focus has been largely on the involvement of the VTA in reward driven behaviour. There is some evidence connecting VTA neuronal activation to the acquisition of conditioned fear, but little evidence that the VTA is involved in conditioned fear extinction. However, Kellett and Kokkinidis (2004) recently demonstrated that stimulation of the amygdala during extinction training retards the extinction of conditioned fear responses. Given that electrical stimulation of either the amygdala or the VTA can have similar effects on behavioural expressions of fear, and electrical stimulation of the



VTA has been shown to sensitize amygdaloid neurons (Gelowitz & Kokkinidis, 1999), it is expected that electrical stimulation of the VTA would also impair extinction learning. Thus, the main hypothesis of this study is that intra-VTA electrical stimulations delivered to rats during fear extinction training will retard the extinction of conditioned fear responses. It is expected that extinction impairments induced by VTA electrical stimulation will mimic extinction impairments observed by Kellett and Kokkinidis (2004) when they used amygdaloid stimulation to interfere with extinction learning. This expectation based on evidence that the mesolimbic pathway between the amygdala and the VTA that plays a vital role in learned fear (Oades and Haliday, 1987; Munroe and Kokkinidis, 1997; Millian, 2003; Nader and LeDoux, 1999; Horvitz, 2000; Giftkins et. al., 2002).

By using Pavlovian fear conditioned rodents, this study aims to investigate the effects on fear potentiated startle (as a measure of fear extinction) following extinction training consisting of 120 non-reinforced presentations of a CS (light) paired with 120 sub-threshold, 1 second, intra-VTA electrical stimulations. The experimental parameters used in the current experiment will be a replication of those used in Kellett and Kokkinidis (2004) experiments, except the stimulation current used to stimulate the VTA will be raised from 300- $\mu$ A to 500- $\mu$ A to account for the VTA being down stream from the amygdala. It is hypothesized that fear-potentiated startle amplitudes will remain elevated in rats that receive intra-VTA electrical stimulation in comparison with control animals, thus reflecting a resistance to extinction.

If an extinction deficit can be induced by intra-VTA electrical stimulation, possible reasons for the deficit will be explored. VTA stimulation could impede the formation of the new CS association required for extinction learning. Conversely, VTA stimulation might evoke a generalized state of arousal making the termination of fearfulness within the training context difficult. Both possibilities potentially augment startle on a test day, but the neural processes underlying augmentation could differ. Thus, comparing the effect of pairing versus randomization of light and electrical stimulation presentations will be essential. So, one group of rats will receive each light

presentation immediately paired with intra-VTA stimulation, and one group will receive a randomized schedule of light and intra-VTA stimulations.

Possible sensitizing effects of VTA stimulation in the absence of extinction training will also be examined. Additionally, the possible aversive effects of stimulating the VTA alone will be explored by replacing footshock with VTA stimulation during the initial fear conditioning process.

Finally, as has been previously outlined, there is much debate regarding differential roles and behavioural effects of dopamine neurons in different regions (Deutch et al., 1985; Mirenowicz & Schultz, 1996; Berrige & Robinson, 1998; Brown & Marsden, 1998; Gurraci & Kapp, 1999; Wise, 2000; Pezze & Feldon, 2004). Because the VTA sits adjacent to and contains A9 dopamine neurons that project to the substantia nigra [SN] (Oades & Haliday, 1987), and the ventral amygdalofugal (VAF) pathway from the central amygdala to the brainstem passes through the SN (Swanson, 1982), the extinction/stimulation experiment will be repeated using SN electrode implanted rats. As previously mentioned, nigrostriatal dopamine system is believed to control procedural aspects of movement and motivation (Hornykiewicz, 1979), because it feeds into the dorsal basal ganglia, which is thought to mediate the acquisition, storage and expression of cognitive and behavioural habits (Alcaro, Huber and Panksepp, 2007). Most research has examined SN dopaminergic regional and cellular functioning in relation to reward learning. Although previous studies have found little effect on fear potentiated startle following either electrical stimulation (Borowski & Kokkinidis, 1996) or 6-OHDA lesions of the SN (Davis, 1992), a possible functional dissociation between the SN and VTA would have exciting implications.

it is hoped that the findings will elucidate the role of the VTA in the manifestation and maintenance of learned fear and/or profound fearfulness, thereby contributing to understanding fear related psychopathologies.

## **2. Materials and Methods**

### **2.1 Subjects.**

A total of 80 naive male albino Wistar rats bred at University of Canterbury from stock originally from Charles River, Quebec, Canada were used. The animals were housed in groups of 4 per cage with food and water available ad-lib. The rats were kept in a climatically controlled environment at  $20 \pm 1$  °C. Animals were maintained on a 12 hr light/dark cycle (lights on 8 A.M.) and were tested during the light portion of the cycle. Prior to surgery the initial weights of the rats ranged from 290 to 380 gms.

### **2.2 Surgery.**

All experiments and surgery complied with animal protocols approved by the University of Canterbury Animal Ethics Committee. Animals were implanted with electrodes in aseptic conditions, anesthetized intraperitoneally (i.p.) with sodium pentobarbital (90 mg/kg). Twenty minutes later an i.p. injection of atropine (.12 mg/kg) was administered to aid respiration. Immediately prior to surgery rats received an analgesic subcutaneous (s.c.) injection of Ketophan (10 mg/kg) below the neck. Animals also received a 0.2ml injection of Mepivacaine (20mg/ml) to the incision site prior to surgery. Rats were placed in a Stoelting stereotaxic apparatus (Wood Dale IL) and the horizontal plane was levelled using landmarks bregma and lambda on the skull surface. Stereotaxic coordinates were based on co-ordinates from a standard stereotaxic atlas of the rat brain (Paxinos & Watson, 1997), and then altered to compensate for the 10 degree lateral angle of the implant. Thus, co-ordinates given are those used at the 10 degree angle and not the equivalent co-ordinate from the atlas, unless otherwise specified. A stainless steel electrode (MS-303/1; Plastic One, Roanoke, VA) was unilaterally implanted

into the ventral tegmental area (AP: -5.0 mm from bregma; L: +/-2.5 mm from the sagittal suture; V: -8.5 mm from the skull surface), or into the substantia nigra (AP: 5.2mm; L: +/- 2.5 mm; V: -8.8 mm). Within each animal group equal proportions of rats were unilaterally implanted with electrodes into either the right or left hemisphere. The electrodes were fixed to the skull using dental cement and 6 stainless steel jeweller's screws (Lomat, Quebec, Canada). The animals were given 7 days to recover before testing took place.

## 2.3 Apparatus.

All experiments were conducted using an acoustic startle system (MED Associates, Fairfield, VT), and associated stimulus generators for light, shock, noise, and electrical brain stimulation were controlled by computer with purpose-designed software. Acoustic startle reflex amplitudes were measured in four identical chambers constructed of melamine (interior dimensions: 600mm wide x 560 mm high x 340 mm deep), in which animals were restrained in movement restrictive cages (16.5 cm long x 8 cm wide x 9 cm high). Interior surfaces of the cages were covered with sound-attenuating acoustical foam to the depth of 25 mm, and constructed of horizontal stainless steel rods 0.25 cm in diameter, that comprised the walls and lid of each cage. Stainless steel rods of 0.45 cm in diameter formed the floor grid, which was attached to a scrambled constant current shock generator. All rods are spaced 15mm apart. A 2.8 watt bulb and a 6.0 cm horn tweeter was situated 10cm back from each startle cage. Each cage was mounted on a MED Associates accelerometer-based transducer platform (250 mm long x 115 mm wide x 45 mm high). In this system movement of the animal is translated into proportional variations in voltage output and is filtered and amplified before being measured by a MED Associates analogue-to-digital converter (ADC) card, which controls presentation of the stimuli.

The acoustic startle stimulus consisted of a 100-ms white noise burst with a rise-decay time of 10 ms, produced by a MED Associates ANL 925

Programmable Audio Stimulator, amplified by an ANL 925A Audio amplifier and presented through the horn tweeter in each chamber. Ambient noise in the chambers was 35 dB as measured by a Simpson (model 860; Elgin, IL) sound level meter (A-scale). The 600- $\mu$ A foot-shock used for fear conditioning was produced by a constant current scrambled shock generator. Electrical brain stimulation into the VTA was generated by constant current stimulators connected to commutators located at the centre top of each chamber with stimulator leads dropping just below the lid of each startle cage. Each 1 second electrical brain stimulation consisted of a 100 Hz train of monophasic square waves (0.1-ms pulse duration). Brain stimulation currents were measured in base-peak units.

## 2.4 Behavioural procedures

### 2.4.1 *Acoustic startle screening*

After a post-operative period of approximately 7 days, all animals were tested for acoustic startle over two consecutive days using 120 noise bursts between 90-98 dB (20-s ISI). An appropriate decibel level that produced average startle amplitude between 100-300 units was selected and used for each rat for all subsequent testing. Once rats were randomly assigned to one of four startle chambers for the acoustic test, the chamber allocation remained the same for each rat to minimize contextual influences on fear behaviour. Rats were also given a habituation time of 5 min whenever they were put in the chamber.

### 2.4.2 *Pavlovian fear conditioning*

Twenty four hours following acoustic screening, animals were fear conditioned using 30 light (CS) + footshock pairings (US) delivered in the startle chamber (inter-trial-interval (ITI) = 56s). The light presentation lasted 3.5s immediately followed by a 0.5s footshock (600  $\mu$ A). To allow animals to acclimatise to the

testing environment each animal was given five minutes habituation time before stimuli presentations. Before conditioning sessions, each cage was thoroughly cleaned and sprayed with 70% ethanol to eradicate any fear-inducing odours that could potentially confound results. Presentation of the CS was kept to a minimum of 5 trials to avoid any possible extinction effects.

#### *2.4.3 Fear potentiated startle testing*

Fear potentiated startle testing was always administered twenty four hours after all manipulative procedures. The first fear test (FPS I) followed fear conditioning and consisted of an initial block of 10 white noise burst trials (30s ITI) to measure baseline startle amplitudes. Rats received a second block of 5-noise burst trials (30s ITI), and then a final block of 5 presentations of the CS (light, 3.5s) followed immediately by a 100Hz train of monophasic noise burst (30s ITI). The second fear potentiated startle test (FPS II) followed extinction training consisted of 20 white noise bursts, 10 noise, and 10 light + noise trials (ITI 30s).

#### *2.4.4 Extinction - VTA/SN electrical stimulation*

Following fear conditioning and FPS test I, fear potentiated startle amplitudes were compared between animals. Animals were then assigned to different experimental groups so that there was an approximately equal distribution of animals that yielded high and low startle amplitudes represented in each group. This was done to try to minimize magnitude variability among baseline startle amplitudes, which could mask differences between experimental conditions. Ten animals were allocated to one of the three groups, each group exposed to different experimental conditions during extinction training. The first group, identified as the paired stimulation (paired) group, and were given 120 light presentations (CS), each presentation paired with a 1s intra-VTA electrical stimulation. The second group, identified as the unpaired

stimulation (unpaired) group received a randomized schedule of 120 light presentations and 120 intra-VTA stimulations, so that the sequence of stimuli presentations was unpredictable. The final group was the non-stimulated group and identified as the control group (control) because they received only 120 presentations of the light (CS) without intra-VTA stimulations. Animals that did not exhibit a conditioned fear response to the CS were eliminated from further experimentation.

Extinction training was conducted 24 hours following the first FPS test. All rats were connected to the stimulators inside the startle chamber and exposed to 120 CS-light (ITI 14s) presentations. For rats in both VTA stimulation groups, animals received a 3.5s presentation of the light (CS) that was either immediately followed by a 1-s electrical stimulation within the VTA of 500  $\mu$ A (base-peak) in the case of the paired group, or, light offset was followed by a 6 second delay preceding either VTA stimulation (500  $\mu$ A; 1s) or another light presentation for the unpaired stimulation group. For the unpaired group the sequence of CS and stimulation presentations was randomized in an effort to control for associative learning effects possibly produced by pairing the CS with the stimulation. The delay between each presentation was always 6 seconds to ensure that rats in both groups were exposed to the experimental context for the same length of time (ITI: 14s). The current level of 500  $\mu$ A (base-peak) was selected because a current level of this magnitude has been previously shown to support intracranial self-stimulation behaviour in rewarding brain stimulation experimental paradigms (Kokkinidis, personal communication, 2005). In addition, previous research within our laboratory has shown that 120 intra-amygdala stimulations using a current level of 300  $\mu$ A (base-peak) is sub-threshold for the induction of after-discharge, leading us to select a the higher current level of 500  $\mu$ A because the VTA is situated downstream from the amygdala (Kellett & Kokkinidis, 2004).

Rats in the control group did not receive VTA stimulation. They were attached to the stimulator leads, but the stimulators were turned off. Thus, control animals received extinction training of 120 non-reinforced presentations of the

light/CS (ITI: 14s). Twenty four hours later, all rats received a second fear potentiated startle test (FPS II) to reassess startle amplitudes following extinction training with/without intra-VTA stimulation. These experimental parameters were repeated using a further 20 rats, but electrical stimulation was delivered to the substantia nigra [SN] instead, and the unpaired condition was also excluded.

#### *2.4.5 VTA stimulation and conditioned fear expression*

To examine the effects of VTA stimulation on normal conditioned fear expression, 16 naive rats were unilaterally implanted with bipolar electrodes into the VTA and then underwent fear conditioning and FPS I testing. The usual gap of 24 hours was given between each experimental component. Instead of extinction training, 24 hours after the first FPS test 8 rats received only VTA 120 1s stimulations at 500  $\mu$ A (base- peak) in their startle chambers, in the absence of the CS. Delivery of the VTA stimulations followed the same temporal parameters as the extinction procedure (14s ISI). The following day, rats were given a second FPS test to examine the effects of VTA stimulation on fear levels. The remaining 8 rats (used as a comparison group) received surgery, and were exposed to exactly the same acoustic startle and conditioning protocols as their counter-parts. But, instead of receiving 120 VTA stimulations these 8 control animals were hooked up to a disabled stimulation commutator and left in a darkened chamber for the same amount of time as the experimental group. The second FPS test 24 hours later presumably demonstrates only temporal effects on the expression of conditioned fear while simultaneously controlling for any restraint and contextual stress incurred during exposure to the experimental apparatus. Thus, this group should display normal levels of fear 24 and 72 hours following conditioning.



#### 2.4.6 *Fear conditioning: VTA stimulation as the US*

To examine the potentially aversive effects of VTA stimulation, footshock was replaced by VTA stimulation as the unconditioned stimulus (US) which was paired with light in the fear conditioning paradigm. Ten surgically implanted rats after acoustic startle evaluation were presented with 3.5-s exposure to the CS immediately followed by 1-s stimulation of 500  $\mu$ A into the VTA across 30 trials (56-s ISI) in one session. Thus, stimulation and CS presentation parameters were consistent with those used during extinction training and the number of CS + US pairings complied with the number of CS + footshock pairings used during normal Pavlovian conditioning. The test for fear potentiated startle (10 baseline trials followed by 5 noise alone and 5 light + noise trials; 30-s ISI) was conducted 24 hours later.

### 2.5 Dependent measures and statistical analysis

For each rat the average startle amplitude scores for baseline noise trials, the last 5 noise trials, and the first 5 light and noise trials were collected from both fear-potentiated startle tests (FPS I & II). The mean startle amplitude for each rat on the second noise trials and the third light + noise trials were pooled into single mean scores as representative of the average startle response to the two different stimulus presentations within the two tests for each experimental condition. Despite all efforts, startle amplitudes on the first test following conditioning, varied greatly. Repeated measures ANOVA analyses were used to examine Test (pre-extinction/stimulation vs. post-extinction/stimulation) x Stimulus (noise alone vs. light + noise) responses, within each group. Simple effects analyses using ANOVA and Student's t-tests were performed on the separate test results within groups, and were also used to examine the differences between group means.

## 2.6 Perfusion and histology

Upon completion of the experiments, rats were killed with an overdose of sodium pentobarbital and perfused intracardially with saline followed by 10% formalin solution. Brains were removed and stored in a formalin solution for approximately 1 week before being transferred to sucrose (70%) solution. After a 2-3 week storage period, coronal slices (40  $\mu\text{m}$ ) were taken and stained with cresyl violet and evaluated under a microscope to verify electrode placements, as per a standard stereotaxic atlas (Paxinos and Watson, 1997).

### 3. Results

A summary of experiments 1- 4 is shown in Table 1 below.

*Table 1:* Table showing design and protocols of the four experiments.

| EXPERIMENT |                | PAV CON   | FPS* I | EXTINCTION TRAINING                 | FPS II |
|------------|----------------|-----------|--------|-------------------------------------|--------|
| 1          | Control (10)** | Footshock | 24 hrs | 120 light, 3.5 s; 14s ITI           | 24 hrs |
| 1          | Paired (8)     | Footshock | 24 hrs | 120 light + stim, 3.5s; 1s; 14s ITI | 24 hrs |
| 1          | Unpaired (7)   | Footshock | 24 hrs | 120 light + stim, 3.5s; 1s; 14s ITI | 24 hrs |
| 2          | Control (8)    | Footshock | 24 hrs | None; cage exposure only            | 24 hrs |
| 2          | Stim (8)       | Footshock | 24 hrs | 120 stim alone, 1s; 14s ITI         | 24 hrs |
| 3          | Stim – US (9)  | VTA Stim  | 24 hrs | N                                   | N      |
| 4          | SN control (8) | Footshock | 24 hrs | 120 light, 3.5 s; 14s ITI           | 24 hrs |
| 4          | SN stim (8)    | Footshock | 24 hrs | 120 light + stim, 3.5s; 1s; 14s ITI | 24 hrs |

\* *Fear potentiated startle test*

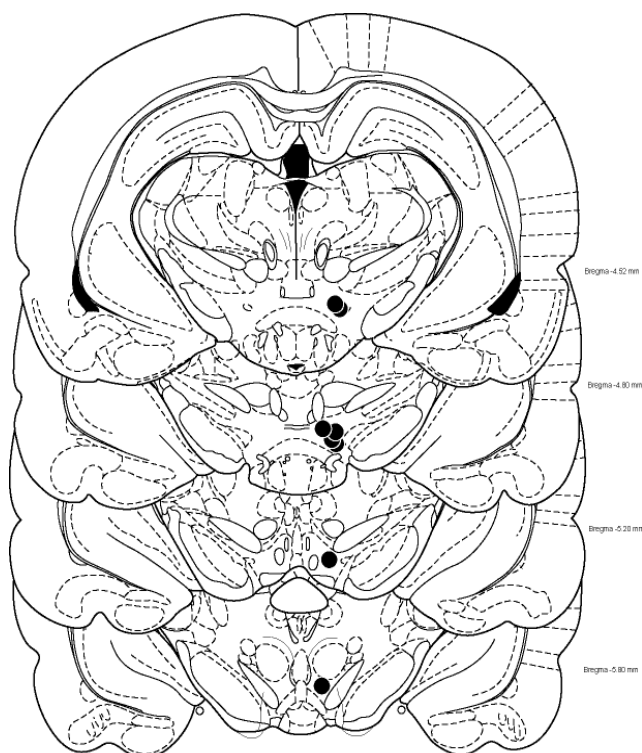
\*\**Number of animals included for statistical analyses.*

#### 3.1 Effects of VTA stimulation on conditioned fear extinction

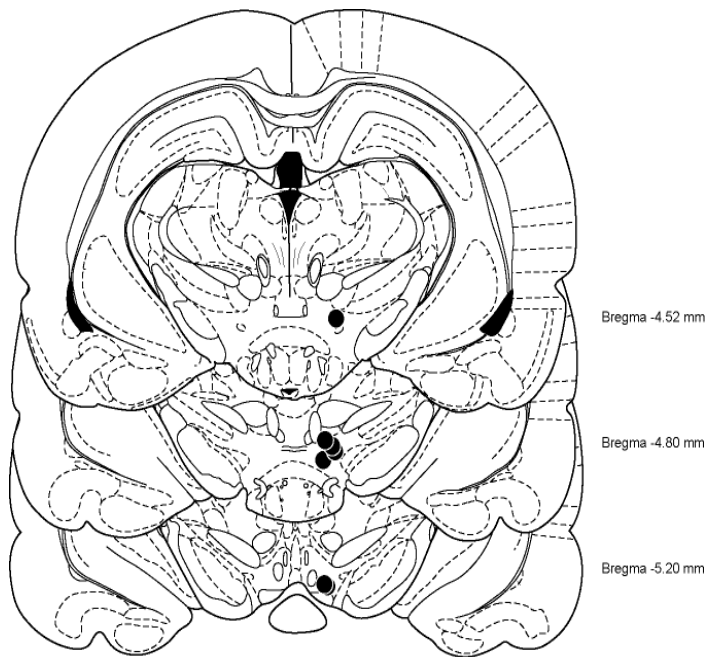
##### 3.1.1 Comparing paired, unpaired and control groups

Of the 30 animals intended for use in this experiment three rats were excluded from the overall analysis due to electrode misplacement. In two rats electrodes were located in the supramammillary area and both showed decreased startle levels on the second fear potentiated startle test following extinction/stimulation; noise alone: 436.4 and 320; light + noise: 223.2 and 293.2, respectively. It is noteworthy that startle levels were not increased in these animals, given that the supramammillary bodies are implicated in the control of fear-related emotive and cognitive functioning (Millan, 2003). The remaining rat had an electrode located in the paranigral nuclei, and this rat

also did not demonstrate fear potentiated startle to the CS on the second test: 377.8 (noise alone) and 152.0 (light + noise). The noticeable decrease in startle amplitude in response to the CS suggests that extinction of conditioned fear was evident in all three animals. As can be seen in Figures 2 and 3, electrode placements for the remaining 15 experimental animals were located within the VTA and these animals included in the statistical analysis.



**Figure 2:** Electrode Placements in the VTA of the paired stimulation group adapted from the Paxinos and Watson (1997) rat brain atlas.



**Figure 3:** Electrode Placements in the VTA of the unpaired stimulation group adapted from the Paxinos and Watson (1997) rat brain atlas.

Across all three stimulation-extinction groups: control, paired and unpaired stimulation, a repeated measures two-way ANOVA [(pre-extinction vs. post extinction) x (noise alone vs. light + noise)] revealed a significant Group x Test x Stimulus interaction,  $F_{(2, 22)} = 4.07$ ,  $p = 0.03$ . This result reveals that patterns of acoustic startle in response to the noise alone and the light + noise presentations varied significantly between the three different stimulation conditions. To determine where this difference occurred, a repeated measures ANOVA was performed on data from the first FPS test alone comparing the three stimulation conditions (control vs. paired vs. unpaired stimulation) and startle responses (noise alone vs. light + noise) as the second factor. Detection of a quantifiable difference between the groups on the first FPS test would indicate that baseline startle amplitudes were too varied to be legitimately compared. A significant main effect for stimulus was revealed,  $F_{(1,22)} = 67.65$ ,  $p < 0.001$ , indicating that light cued startle responses were significantly greater than startle responses to noise alone. There was no significant Stimulus x Stimulation condition interaction,  $F_{(2, 22)} p = .31$ . As can be seen in Figure 4, mean startle amplitudes differences ([light + noise] – [noise alone]) were of a similar magnitude across all three groups, indicating

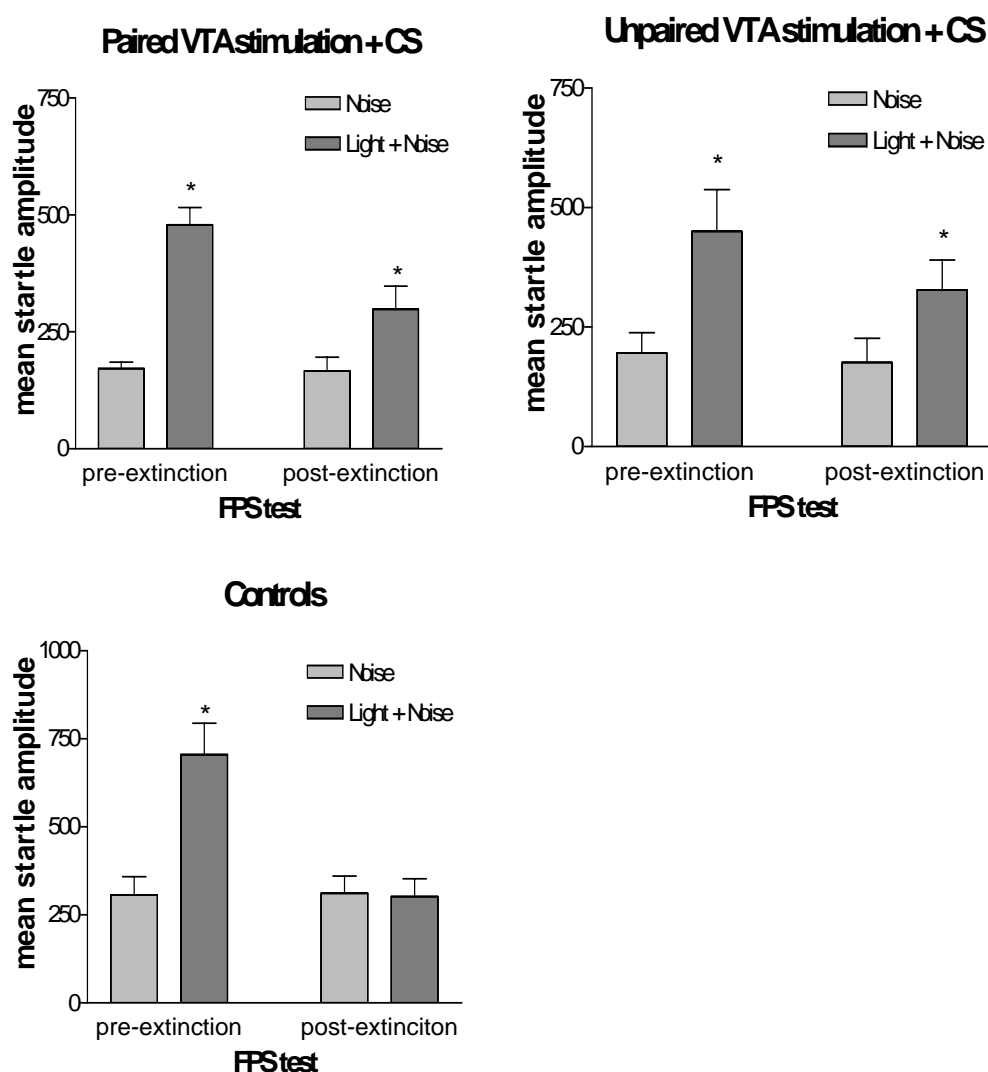
that comparable levels of baseline conditioned fear had been achieved.

However using FPS II data only, a 3 x 2 ANOVA ([no-stimulation vs. paired vs. unpaired stimulation] x [noise alone vs. light + noise]) with repeated measures on the second factor revealed a significant interaction (Stimulation Condition x Stimulus)  $F_{(1, 22)} = 5.73$ ,  $p = 0.009$ , and a significant main effect of stimulus  $F_{(1, 22)} = 17.17$ ,  $p < 0.000$ . This finding indicates a significant variation in fear potentiated startle amplitudes among the different stimulation conditions. A simple effects analysis revealed a significant increase in mean startle amplitude to the light + noise compared to the noise alone for the paired stimulation condition,  $F_{(1, 7)} = 14.75$ ,  $p = 0.006$ . Rats in the unpaired stimulation condition demonstrated startle amplitudes that were also significantly greater in response to light + noise vs. noise alone  $F_{(1, 6)} = 16.15$ ,  $p = 0.006$ . Importantly, mean startle amplitudes to both stimuli conditions for the control group did not differ significantly,  $F_{(1, 9)} = .819$ ,  $p = .819$ . This latter result clearly demonstrates that conditioned fear responding was extinguished in control animals following 120 non-reinforced presentations of the light. These results are presented in Figure 4 and show average startle amplitudes for each stimulus condition (noise alone and light + noise) for both FPS tests (post-conditioning vs. post-extinction) for each stimulation condition (paired, unpaired, and no stimulation). From the presented results it can be readily interpreted that electrical stimulation of the VTA during extinction training induced an extinction learning deficit.

It is possible that VTA stimulation induced a sensitization of the acoustic startle response rather than a specific extinction learning deficit. Thus, the first 20 noise post-conditioning trials were compared with the first 20 noise post-extinction trials using repeated measures 3 x 2 ANOVA (Test x Stimulation condition). There was no significant interaction between the two factors,  $F_{(2,22)} = 1.79$ , n.s., but a significant main effect for Test was detected,  $F_{(1,22)} = 13.27$ ,  $p = 0.001$  because, across all three stimulation conditions, the average acoustic startle amplitudes dropped significantly on the second test compared to the first test. Therefore, 120 1-s electrical stimulations delivered

to the VTA did not sensitize acoustic startle responses. Additionally, because initial baseline acoustic levels were not seen to elevate on the second FPS test it can also be assumed that VTA stimulation did not evoke a generalized conditioned fear to the testing/training context.

### **Effect of VTA stimulation on extinction learning**



**Figure 4.** Effects of VTA stimulation on extinction learning. Mean (+/- S.E.M.) startle amplitudes for noise vs. light+noise on each FPS test (pre-extinction vs post-extinction) are compared between groups receiving 120 VTA stimulations paired (N= 8) and unpaired (N= 7) with light presentations and controls (N= 10) receiving extinction training only. Startle amplitudes displayed by control animals do not differ between the two stimulus conditions on the second fear potentiated startle test following extinction training. Animals that received VTA stimulation during extinction training (paired and unpaired) still show significantly augmented startle responses to light + noise stimuli compared to noise alone on the second FPS test (\*  $P < 0.05$ ), indicating an extinction learning deficit. Refer to Figures 2 & 3 for electrode placements in VTA stimulated animals.

In summary, the main finding from this experiment was that electrically evoked activation of the VTA produced a generalized enhancement of fear arousal during the extinction training session that ultimately had a detrimental effect on inhibitory fear learning, which could not be specifically attributed to associative learning processes.

### 3.2 VTA stimulation and the possible sensitization of conditioned fear expression

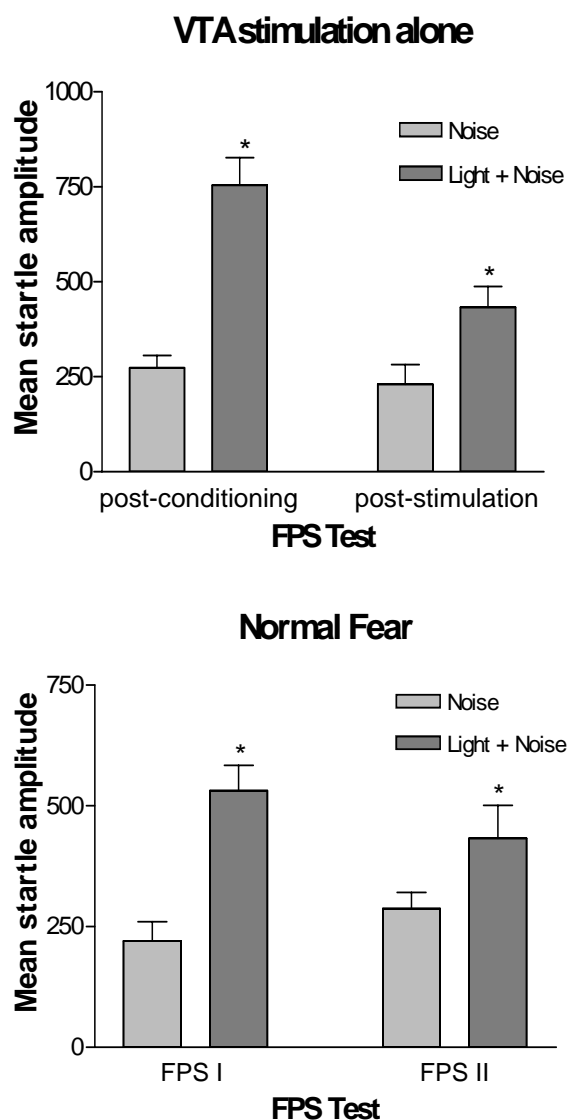
There are a few possible explanations for the observed extinction deficit following VTA electrical stimulation during extinction training. One such possibility is that the 120 1-s, sub-threshold electrical stimulations delivered to the VTA sensitized the pathway from the central amygdala to the PnC (Rosen & Davis, 1988), artificially elevating acoustic startle, and appearing behaviourally as an enhancement of fear expression rather than an extinction learning deficit. This is a reasonable explanation given that previous research has shown that electrical stimulation of the VTA promotes electrical activity within the amygdala (Kokkinidis, 1992; Gelowitz & Kokkinidis, 1999) and can enhance acoustic startle amplitudes (Rosen & Davis, 1988; Borowski & Kokkinidis, 1996). To test this possibility 18 electrode implanted rats that had been successfully fear conditioned, were put in a darkened chamber, hooked up to stimulator leads, but only half the animals (9) were stimulated within the VTA 120 times (1s per stimulation). The effect of intra-VTA stimulation on conditioned fear expression was compared to normal levels of conditioned fear expressed by sham implanted, non-stimulated animals, as shown in figure 5.

Of the 18 animals implanted, 1 rat did not complete the experiment due to head-cap loss, and was eliminated from the analysis. Another rat was excluded from the analysis due to electrode placement being immediately dorsal to the VTA lying between the substantia nigra compacta and the zona incerta. This animal displayed a complete lack of conditioned fear responding

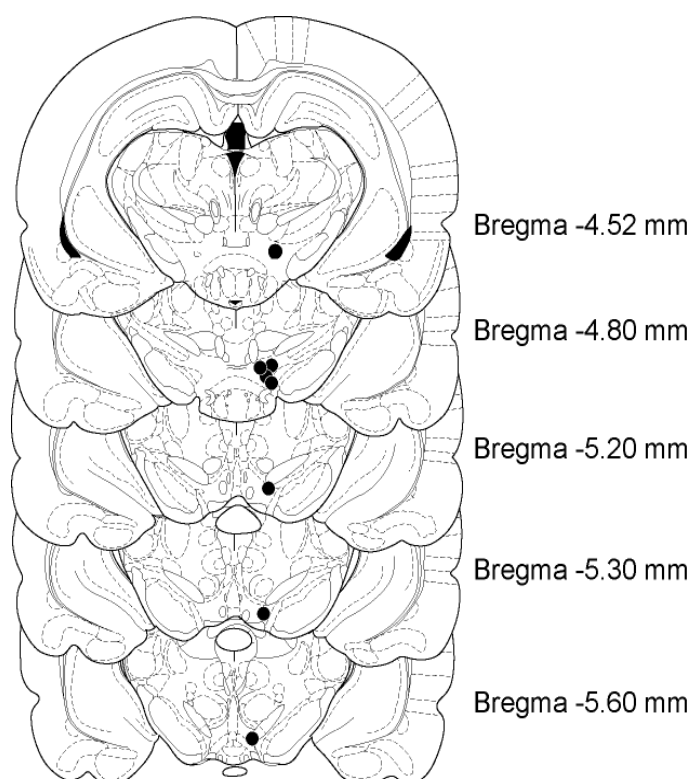


to presentations of the light on the second FPS test, with a mean startle amplitude of 489.0 to the noise alone and 263.4 to the light + noise. It is unclear whether this animal had no recollection of the former CS-US association following the stimulations, or whether conditioned fear had rapidly extinguished due to the 5 + 5 non-reinforced light presentations used as the testing stimulus. Electrode placements for the remaining experimental animals are depicted in Figure 6, with all eight electrodes being located within the VTA.

### **Effect of VTA stimulation alone on previously acquired conditioned fear**



**Figure 5.** Effects of VTA stimulation on previously acquired conditioned fear compared to normal levels expressed by non-stimulated animals. Graphs depict mean ( $\pm$  S.E.M.) startle amplitudes for noise vs. light +noise on each FPS test (post-conditioning vs post-stimulation for experimental group). VTA stimulation alone group received 120 1-s stimulations instead of extinction training 48 hours after Pavlovian conditioning ( $N= 8$ ), and shams received cage exposure only without stimulation or extinction training ( $N= 8$ ). On both FPS tests startle amplitudes were significantly higher in response to light + noise trials compared to noise alone trials ( $* p < 0.05$ ). However, the pattern of startle amplitudes in response to differing stimuli conditions did not vary significantly between the two stimulation conditions, indicating that 120 VTA stimulations did not sensitize conditioned fear responses above normal levels.



**Figure 6.** Schematic represents electrode placements in VTA stimulated, non-extinguished animals, with co-ordinates taken from the Paxinos and Watson (1997) rat brain atlas.

A 3 x 2 repeated measures ANOVA was used to compare stimulated vs. non-stimulated on within-group factors: Test (FPS I vs. FPS II) x Stimulus (noise alone vs. light + noise). The results indicated that there was no significant variation between the groups in within-groups measures. The main effect for Group was not significant,  $F_{(1, 14)} = 1.64$ , n.s., and there was no significant interaction between Group x Test x Stimulus factors  $F_{(1, 14)} = 1.21$ , n.s., or Group x Stimulus  $F_{(1,14)} = .09$ , n.s., or Group x Test  $F_{(1, 14)} = .08$ , n.s.; all of which would suggest that 120 VTA stimulations did not significantly affect normal levels of fear. As would be expected, there was a significant main effect for stimulus  $F_{(1,14)} = 83.59$ ,  $p < 0.000$ , indicating that conditioned fear responding was maintained between the two tests as acoustic startle significantly increased in response to light + noise vs. noise alone. There was also a significant main effect on the Test factor,  $F_{(1, 14)} = 4.69$ ,  $p = 0.047$ . The interaction between Test x Stimulus was also significant,  $F_{(1, 14)} = 18.84$ ,  $p < 0.000$ , indicating that startle amplitudes varied significantly between the two

different tests, and in response to the two different stimuli.

As can be seen in Figure 5, baseline startle amplitudes remained relatively stable with no significant difference at  $p < 0.05$  between the two tests detected. Startle amplitudes in response to the light + noise did drop; hence the barely significant ( $p = 0.047$ ) reduction in fear potentiated startle amplitude on the second test compared to the first for both stimulation conditions. Nevertheless, a repeated measures ANOVA comparing the data from the second FPS test (Stimulus: noise alone vs. light + noise) for non-stimulated vs. stimulated animals yielded a significant main effect for stimulus  $F_{(1, 14)} = 32.39$ ,  $p < 0.001$ , with no significant Group x Stimulus interaction,  $p = .368$ . In both stimulation conditions, conditioned fear responses were definitely evident on the second fear potentiated startle test. Thus, fear potentiated startle augmentation was not significantly greater than normal levels of fear. Overall, these results negate any proposition that intra-VTA stimulation parameters used in the current experiments were sufficient to exaggerate conditioned fear responding or sensitize the expression of generalized fearfulness.

### 3.3 Fear conditioning: VTA stimulation as the unconditioned stimulus

Another alternative explanation for the resultant extinction deficit is that the stimulation itself could have an aversive effect on the animals. If VTA stimulation has inherent unconditioned fear arousing properties, then pairing VTA stimulation with the light (CS) simply reconditions animals during extinction training. Thus, impairment of extinction learning could be attributed to heightened emotional arousal induced by electrical stimulation of the VTA that maintained rather than reduced conditioned fear responding.

To examine this possibility, VTA electrode implanted rats were conditioned

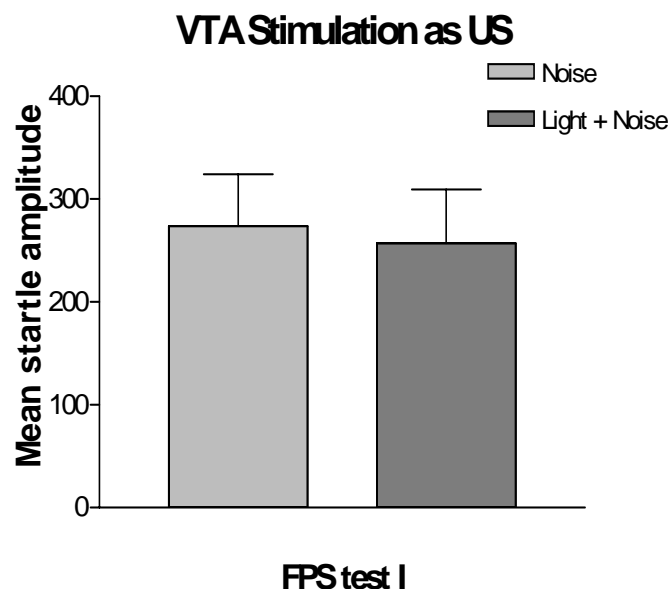
using light paired with VTA stimulation as the US, rather than footshock. After 30 within-session pairings of VTA stimulation and light, animals were tested for fear-potentiated startle. Previous findings from a preliminary investigation (unpublished, 2004) conducted in our laboratory suggested that VTA stimulation might contain aversive properties because experimental animals displayed conditioned fear responding akin to conditioned fear responding that has been observed when using footshock as the US. However, only 6 animals were involved and their fear responses were inconsistent, indicating the need to replicate the experiment using a greater number of subjects and more rigorous procedures to eliminate possible confounding contextual influences.

Figure 8 depicts a graphic representation of the behavioural results and electrode placements of the 9 animals used in the current experiment. One animal was not included in the analysis due to very low startle amplitudes on the noise alone trials (suggesting deafness), namely mean startle amplitudes for noise alone: 40.2 vs. light + noise: 266.8. In addition to unreliable startle responses, electrode implantation was located within the rostral interstitial nucleus. Two additional animals were excluded due to electrode misplacement. One electrode was located in the deep mesencephalic nuclei and this rat exhibited slightly increased average startle amplitudes to the light + noise: 247.2 vs. 219.4 for the noise alone trials. This area represents a cluster of fibres that are thought to transmit startle-enhancing effects from the amygdala to lower brainstem structures, and therefore might be important in the formation of fear conditioned responses (Koch and Schnitzler, 1997). The other excluded animal also exhibited elevated fear potentiated startle; noise: 218.6 and light + noise: 330.8 with the electrode located definitively within the substantia nigra compacta.

A Student's t-test on post-conditioning test data showed average startle responses to the noise alone: 273.7 (S.E.M: 50.4) was not significantly different from average startle responses to the light + noise: 257.3 (S.E.M: 52.1),  $t_{(8)} = .517$ ,  $p = 0.61$ . In fact, there was a slight decrement in startle

amplitudes on light + noise trials, which is evident in figure 7. Given that there is no evidence of conditioned fear in these animals, it cannot be concluded that VTA stimulation promoted unconditioned fearfulness because 30 stimulations were not aversive enough to act as an effective US in the Pavlovian conditioning procedure. Therefore, it is unlikely that the extinction deficit observed in the main experiment was simply a function of re-conditioning the animals.

### **Pavlovian fear conditioning using VTA stimulation as unconditioned stimulus**



**Figure 7.** Graph depicts mean startle amplitudes to noise alone vs. light + noise which was not significantly different ( $p < 0.05$ ) on FPS test following Pavlovian fear conditioning using 30 VTA stimulations as the US immediately paired with presentations of the light (CS). These results suggest that VTA stimulation does not promote unconditioned fear arousal, and therefore it is unlikely that animals were simply re-conditioned during extinction training (\*  $p < 0.05$  relative to noise alone trials).

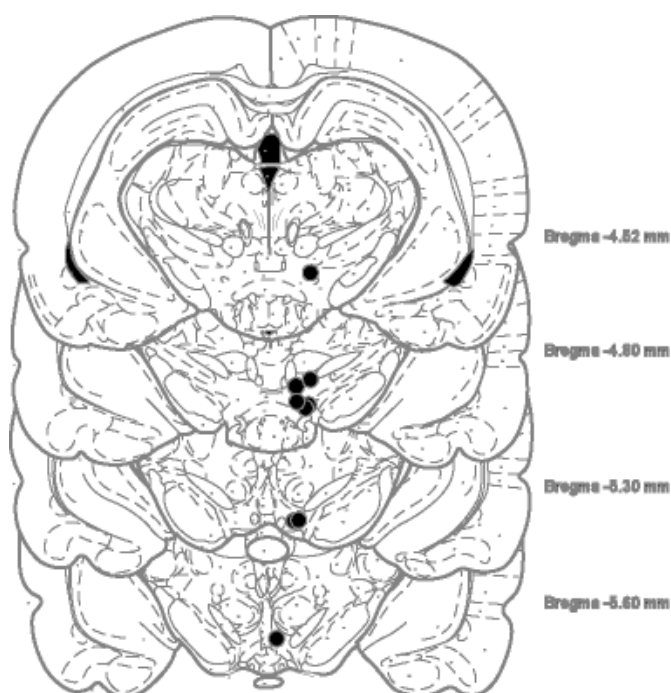


Figure 8. Schematic representation of electrode placements within the VTA in rats, adapted from the Paxinos and Watson (1997) rat brain atlas.

### 3.4 Effects of intra- substantia nigra stimulation on extinction learning

Finally, the main experiment was replicated replacing VTA stimulation with 120 intra-substantia nigra stimulations paired with non-reinforced presentations of the light. By applying electrical stimulation to the substantia nigra, which is immediately adjacent to the VTA, it was hoped we could examine whether the effects of stimulation on extinction learning were regionally specific. All electrode placements for the 8 experimental animals were satisfactorily located in the substantia nigra pars reticulata and a schematic representation of implantation locations can be seen in Figure 10.

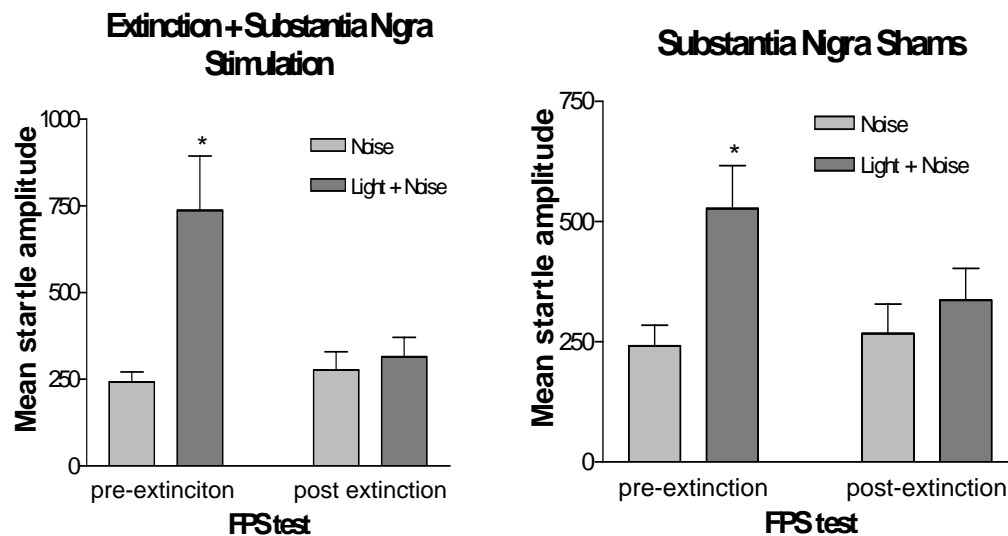
A two way repeated-measures ANOVA ([Test: FPS I vs. FPS II] x [Stimulus: noise alone vs. light + noise]) comparing Stimulation conditions: substantia nigra stimulation vs. shams, revealed a non-significant interaction between factors: Stimulation condition x Test x Stimulus,  $(1, 15) = 2.97$ ,  $p = .10$ . Furthermore, interactions between Stimulation condition x Test and Stimulation condition x Stimulus were also not significant,  $F_{(1, 15)} = 1.08$ , n.s.

and  $F_{(1, 15)} = .52$ , n.s., respectively. These non-significant results represented in Figure 9 demonstrate that the pattern of startle in response to noise alone vs. light + noise on both FPS tests was markedly similar across both stimulation conditions (substantia nigra stimulation vs. sham).

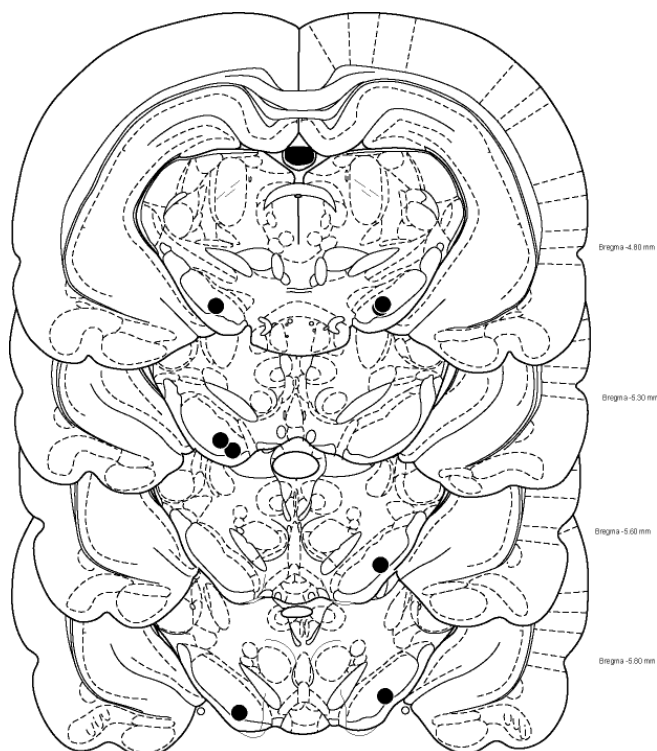
The interaction between Test x Stimulus factors was significant,  $F_{(1, 15)} = 23.29$ ,  $p = 0.01$ , indicating that the pattern of startle responses to noise alone vs. light + noise was significantly different on the post-conditioning compared to the post-extinction FPS tests. Both main effects for Test and Stimulus factors were also significant,  $F_{(1,15)} = 6.65$ ,  $p = 0.02$  and  $F_{(1, 15)} = 13.118$ ,  $p = 0.02$ , respectively. These results would be expected if conditioned fear responses are successfully extinguished in animals following extinction training. Student's t-tests used to analyse simple effects to confirm startle amplitude differences reflected successful conditioning and extinction. The results depicted in Figure 9 show that for S/N stimulated subjects, mean startle amplitude on light + noise trials ( $635.75$  [ $\pm 135.13$ ]) s was significantly higher than baseline startle amplitude ( $230.27$  [ $\pm 28.98$ ]) on the post-conditioning FPS test,  $t_{(7)} = -3.10$ ,  $p = 0.01$ . This was not so for the FPS post-extinction test with the mean startle amplitude on light + noise trials ( $275.57$  [ $\pm 44.5$ ]) being not significantly different from baseline startle amplitude ( $277.4$  [ $\pm 59.03$ ]),  $t_{(7)} = .041$ ,  $p = .96$ .



### Effect of substantia nigra stimulation on extinction learning



**Figure 9.** Effect of 120 intra-substantia nigra stimulation on extinction learning in comparison to control animals. Graph depicts successful Pavlovian conditioning in both stimulation conditions, whereby mean startle amplitudes light + noise were significantly higher than noise alone startle amplitudes ( $\pm$  S.E.M) on FPS I test following Pavlovian fear conditioning ( $p < 0.05$ ). No significant difference was detected between noise alone vs. light + noise for both groups on the second FPS test following extinction training. By comparison to control animals these results indicate that S/N stimulation had no effect on the extinction of learned fear.  
(\*  $p < 0.05$  relative to noise alone trials)



**Figure 10.** Schematic representation of electrode placements located within the substantia nigra, adapted from the Paxinos and Watson (1997) rat brain atlas.

Likewise, mean startle amplitude on light + noise trials for sham subjects (527.15 [ $\pm$ 89.15]) was significantly greater than baseline startle levels (241.5 [ $\pm$ 43.2]) on the post-conditioning FPS test,  $t_{(7)} = -3.26$ ,  $p = 0.01$ . There was no significant difference between baseline mean startle amplitude (267.42 [ $\pm$ 60.8]) and mean light + noise startle amplitude (336.8 [ $\pm$ 65.8]) on the post-extinction FPS test,  $t_{(7)} = -.88$ ,  $p = .40$ . Despite the appearance that, on average, S/N-stimulated subjects exhibited fully extinguished conditioned fear following extinction training but shams showed traces of conditioned fear, a Student t-test on difference scores ([light + noise] – [noise alone]) on the post-extinction FPS test showed that there was no significant difference between the two groups,  $t_{(15)} = .85$ ,  $p = .41$ . These results indicate that electrical stimulation to the substantia nigra region (which is immediately adjacent to the VTA), when paired with non-reinforced CS during extinction training had no observable effect on extinction learning. Startle responses were suppressed in response to CS presentations, indicating that learned fear response was extinguished.

A comparison between the VTA stimulation/extinction group and the SN/extinction group would have been ideal, but unfortunately vastly different baseline startle amplitudes made this comparison problematic.

## 4. Discussion

### 4.1 Summary of key findings and issues

#### 4.11 *Intra-VTA electrical stimulation during extinction training produced an extinction learning deficit*

It is well known that repeated non-reinforced CS presentations extinguish Pavlovian conditioned fear responses, and this new, neutral association with the CS masks the former aversive, conditioned association (Bouton, 1993). The ability to stop fearfulness in an environment that is no longer threatening is a normal, adaptive response. Recently, it has been suggested that a malfunction of neural processes that govern learned fear might be linked to abnormal fearfulness and anxiety disorders (Cain et al., 2003; Jovanovic et al., 2005). But, the neurobiological substrates that mediate the inhibition of fear are yet to be determined. It is commonly thought that higher neural regions that inhibit the actions of lower regions such as the midbrain, could govern extinction. This presupposes that all learning is a top-down process. For example, prefrontal cortical regions can reduce the firing rate of subcortical neurons in the lateral amygdala, which mediate conditioned fear (LeDoux, 2000; Morgan et al., 1993; Quirk et al., 2003; Rosenkranz et al., 2003). However, laboratory research at the University of Canterbury suggests that disruptions along neural pathways originating at the midbrain level interfere with inhibitory neural connections formed within higher cortical regions. Thus, some forms of learning, particularly fear related learning, may follow a bottom-up trajectory.

The current study examined the effects of electrical excitation of VTA neurons during extinction training after rats had been successfully fear conditioned, using light paired with footshock. The key finding was that in comparison to control animals, electrical activation of the VTA during extinction training—consisting of 120 non-reinforced CS presentations—created an inability to inhibit conditioned fear responding that ultimately impaired extinction learning substantially.

Kellett and Kokkinidis (2004) reported extinction retardation following intra-amygdala stimulation (300  $\mu$ A base-peak; 1-s,) paired with 120 non-reinforced CS presentations. A similar extinction deficit using intra-VTA instead of intra-amygdala stimulation was predicted because research has shown that the VTA plays an important role in the generating emotional responses to aversive stimuli (Guarraci & Kapp, 1999; Horowitz, 2000; Pezze & Feldon, 2004). Furthermore, it has been successively demonstrated that the dopaminergic connection between the VTA and the amygdala is critical to the acquisition of learned fear (Borowski & Kokkinidis, 1996; 1999; Munro & Kokkinidis, 1997; Fendt & Fanselow, 1999; Guarraci & Kapp., 1999; Nader & LeDoux, 1999; LeDoux, 2000; Greba et al., 2001; Maren, 2001). Moreover, pharmacological or electrical activation of both the amygdala and the VTA exaggerate fear responses, whereas lesions to either region can eliminate fear responding (Borowski & Kokkinidis, 1996; Rosen et al., 1996; Kalynchuk, 2000). Based on the current finding and past evidence, it can be inferred that dopamine neurons of the VTA modulate the actions of amygdaloid neurons in the maintenance of learned fear responses (Munroe and Kokkinidis, 1997; Millian, 2003; Nader and LeDoux, 1999; Horvitz, 2000; Giftkins et. al., 2000). Thus, the ability to inhibit conditioned fear responding is hindered by the electrical activation of the mesolimbic system originating in the VTA, leading to an extinction deficit that was similar to extinction deficits produced by amygdala stimulation (Kellett and Kokkinidis, 2004).

Following substantiation of the main hypothesis—that intra-VTA electrical stimulation during extinction training produced an extinction learning deficit—four subsequent experiments were conducted, aimed at isolating the specific effect of electrical VTA activation.

#### 4.1.2 *Eliminating a possible sensitisation effect*

Past research has shown that repeated electrical stimulation of the VTA promotes exaggerated fearfulness (Stevens & Livermore, 1978) and elevates startle in animals (Borowski & Kokkinidis, 1996). Such exaggerated fear

responses have been attributed to a sensitisation of ascending mesolimbic dopaminergic pathways originating in the VTA and projecting to the central and basolateral amygdala (Oades and Halliday, 1987; Guarraci and Kapp, 1999; Gelowitz & Kokkinidis, 1999). If electrical stimulation of the amygdala can augment startle via CE amygdaloid activation of the PnC—the critical locus of the startle circuit—(Koch & Schnitzler, 1997) then it was also probable that 120 intra-VTA stimulations could have the same effect on fear-potentiated startle.

However, fear-conditioned rats that did not receive extinction training exhibited stable fear-potentiated startle amplitudes after 120 (1s) intra-VTA stimulations. These startle amplitudes were no greater than the normal levels of fear exhibited by control animals. In fact, for all VTA stimulated animals (extinguished and non-extinguished) potentiated startle amplitudes were not significantly depressed relative to initial conditioned fear levels. These findings indicate that VTA stimulation did not sensitise auditory startle circuitry, or the behavioural expression of fear. Therefore, fear-potentiated startle was a valid measure of extinction deficits. The possibility that neural damage was incurred by electrical stimulation causing memory loss must be considered; however, the observation that all VTA-stimulated animals remained fearful of the light (CS) suggest that memory loss is not a confounding factor in these findings.

#### 4.1.3 *Eliminating the possibility that intra-VTA stimulation was aversive*

A second explanation for extinction deficits could have been that intra-VTA stimulation of 500  $\mu$ A for 1-second could have aversive effects consequently creating subjective feelings of fearfulness in the rats. In effect, animals that received intra-VTA stimulation during extinction training might have gained a further session of Pavlovian conditioning instead. This was a reasonable possibility given that VTA stimulation has excitatory effects on the periaqueductal grey (PAG), via dopamine and non-dopamine VTA fibres

innervating the PAG (Oades & Halliday, 1987; Gifkins et al., 2002). The PAG is believed to mediate unconditioned expressions of fearfulness involving autonomic, reflexive and stereotyped behavioural responses (Millan, 2003; Di Scala et al., 1987). In addition, preliminary investigations undertaken by the author (Taylor and Kokkinidis, unpublished) suggested that intra-VTA stimulation did have aversive effects on the animals. But the group size was small and no conclusions were drawn.

However, the present experiment had greater subject numbers and demonstrated that VTA stimulation (500  $\mu$ A, 1-second) was an ineffective unconditioned stimulus when it replaced footshock in the Pavlovian conditioning procedure. Thirty CS + US pairings were used, which experience suggests was a sufficient sample to produce conditioned fear in rats. It was noted that surgical skills and head-cap construction vastly improved with experimenter experience. As techniques were refined the animals recovered more quickly and appeared to be less affected by the intervention. Therefore it is likely that some animals in the previous preliminary experiment were exhibiting an unconditioned response to a painful procedure as opposed to reacting to the neurological effects of electrical stimulation. Thus, procedural competency became an important consideration when interpreting results.

In summary, neither individual stimulation of 500  $\mu$ A nor the administration of 120 successive intra-VTA stimulations produced an unconditioned fear response in the rats studied. This implies that the neutral stimulus (CS) was rendered meaningless as a predictor on test day. Nevertheless, it still remains unclear why extinction deficits were produced by intra-VTA stimulation. Two possible explanations are proposed, either intra-VTA stimulation interfered with the neural and/or cellular processes governing the acquisition of extinction learning, or intra-VTA stimulation reinforced the former conditioned association by re-triggering the initial conditioned fear association. Behaviourally, neither alternative is mutually exclusive as both result in the disinhibition of condition fear responding.

#### 4.1.3 *Explanation 1: VTA stimulation directly effects extinction learning processes*

If VTA stimulation had a specific effect on the formation of new extinction associations then VTA stimulation immediately paired with CS presentations might create a greater extinction deficit than a randomised stimulation-CS schedule. One possible cellular explanation for this was that the firing of VTA derived dopamine cells, which are normally inhibited when the CS is not explicitly paired with the US (Collins and Pare, 2000), was disinhibited by VTA stimulation paired directly with CS presentations. However, the results show that neither explicit pairing nor randomization of stimuli presentations differentially affected extinction learning in a quantifiable way. Therefore, VTA stimulation asserted a more generalized effect on behaviour.

In contrast, Milad and Quirk (2002) demonstrated that paired, not pseudorandom, medial prefrontal cortical (mPFC) electrical stimulation and CS (light) presentations during extinction sessions improved extinction learning. The improvement was even greater in the paired group than control animals that received extinction training only (Ibid, 2002). Studies have shown that mPFC integrity and activation is critical to the suppression of conditioned fear responding or extinction learning (Gottfried and Dolan, 2004; Phelps et al., 2004; Morgan et al., 1993; Morgan and LeDoux, 1999; Morrow et al., 1999; Quirk et al., 2000; Myers and Davis, 2002; but see Gerwitz, et al., 1997). It is interesting to compare the two regions because even though VTA pre-extinction lesions have been shown to retard fear extinction (Borowski and Kokkinidis, 1996), results from this study suggest it is unlikely that VTA stimulation directly influences inhibitory learning processes. Instead, based on the present findings, it can be inferred that regulated activity of VTA neurons is required to maintain arousal at an optimal level for fear extinction learning to occur.

One caveat to the comparison between the Milad and Quirk (2002) study and this study, is that the former used freezing as their measure of conditioned

fear responding as opposed to fear-potentiated startle. Methodological differences can differentially affect results. But emerging evidence from subsequent studies suggests an inverse relationship between the VTA and the mPFC, indicating a functional dissociation between the two regions (Barrett et al., 2003). For example, the greatest metabolic activity occurs within the mPFC and not the VTA during extinction learning, whereas the opposite is true during fear conditioning (Ibid, 2003). Another study has shown that low stimulation of the PFC activated GABA neurons in the Acb and the VTA, which in turn inhibited dopamine cellular firing (Sesack & Carr, 2002). These studies add further support for the hypothesis that VTA dopamine neurons are under tonic GABA influence (Paladini et al., 1999), which enables the suppression of dopamine excitation permitting fear inhibition. It could also be proposed that neural impetus of extinction learning comes from the top, down (Carr & Sesack, 2000).

Conversely, the neural processes underlying conditioned fear may follow a bottom-up trajectory. Elevated arousal levels mediated by dopamine pathways originating in the VTA could be required to acquire excitatory associative learning (Borowski and Kokkinidis 1998). In the future it will be important to repeat the current experiments using intra-mPFC electrical stimulation. Demonstrating a functional dissociation between these two regions would support the view that bottom-up neural processes underlie emotional motivation and learning.

#### 4.1.4 *Explanation 2: VTA stimulation reinforces initial CS-US association*

It is possible that intra-VTA stimulation reinforced a pattern of fear conditioned responding by activating dopaminergic mesolimbic pathways. This activation might re-trigger stimulus-affect memory believed to be supported by the amygdala (Kesner, 1998; Lee, Walker and Davis, 1996; Gaffan, 1992; Kellett and Kokkinidis, 2004). In the current study, it is reasonable to assume that dopamine neurons were activated, given that approximately 70% of the cells within the VTA of a rat brain are dopaminergic (Oades & Halliday, 1987). And,



elevated release of dopamine in the Acb and increased firing rates of dopamine neurons in the VTA have been shown to occur in response to aversive or stress-inducing events (Trulson & Preussler, 1984; Tidey and Miczek 1996; Imperto et al., 1992; Horvitz, 2000). Plus, systemic administration of psychomotor stimulants promoting dopaminergic transmission blocks extinction of conditioned fear (Borowski & Kokkinidis, 1996). These findings indicate the involvement of dopamine in learned fear and therefore it is likely that VTA stimulation used in the current study activated mesolimbic dopaminergic pathways.

It has been suggested that the amygdala could be the storage site for all emotional memories, including both reward and fear representations (Borowski and Kokkinidis, 1999; Lee, Walker and Davis, 1996; Gaffan, 1994). However, theories regarding how memory is organized in the brain remain enthusiastically debated (Tulving and Schacter, 1990; Squire, 1992; McDonald and White, 1993; Gaffan, 1994; Eichenbaum, 1995; Squire and Zola, 1998). But, there is the possibility that access to affect memories could be dependent on dopaminergic induced-arousal originating in the VTA (Borowski and Kokkinidis, 1999). For example, either electrophysiological or pharmacological excitation of dopamine neurons within the VTA activates amygdaloid neurons and triggers fearfulness, presumably governed by the amygdala (Stevens and Livermore, 1978; Davis, 1992; Borowski, and Kokkinidis, 1996; 1998; Nader and LeDoux, 1999; Gelowitz and Kokkinidis, 1999; Horvitz, 2000; Gifkins et al., 2002; Millan, 2003). And, increased dopaminergic metabolism and firing activity has been located in both the amygdala and the VTA in response to fear conditioned stress (Deutch, et al., 1985; Guarraci & Kapp, 1999). It is also possible that VTA activation during extinction training sessions interfered with learning processes by reactivating excitatory fear pathways that directly oppose inhibitory neural processes. Based on findings from cellular studies it was recently proposed that inhibitory long-term potentiation (LTP) might be crucial for fear extinction (Bauer and LeDoux, 2004). Inhibitory LTP is thought governed by inhibitory inter-neurons within the lateral amygdala, which suppress the induction of

excitatory LTP and promote inhibitory transmission (Sigurdsson, et al., 2007). As one of the main excitatory neuromodulators, dopamine is believed to be important in gating this inhibitory transmission (Bissiere et al., 2003). Therefore, intra-VTA stimulation of rats used in this study possibly suppressed inhibitory transmission by strengthening excitatory synaptic mechanisms on lateral amygdaloid neurons, which underlie the association between the CS and the US (Maren, 2005). Hence, the possible re-triggering of conditioned fear memories thought to be supported by the amygdala and implicated in fear-related psychopathologies such as PTSD. This would also explain extinction retardation following amygdaloid stimulation during extinction training discovered by Kellett and Kokkinidis (2004), and which inspired this study.

This cellular explanation is also consistent with the finding that intra-VTA stimulation paired with the CS was not sufficient to Pavlovian condition the animals. For the CS to independently drive central amygdaloid mediation of fear expression, a very strong unconditioned stimulus input—like footshock—administered simultaneously with CS input on lateral amygdaloid neurons is required to cause a strong post-synaptic depolarization (Morgan et al., 1993; Collins and Pare, 2000; Le Doux, 2000; Maren, 2001; Sigurdsson, et al., 2007). From this study, it is evident that 30 intra-VTA stimulations of 500  $\mu$ A plus 30 light presentations did not create a strong enough CS+US convergence, meaning that excitatory LTP did not occur, and therefore, animals remained unconditioned.

In summary, the preferred interpretation of the current results is that VTA stimulation reinforced the association between the conditioned and unconditioned stimulus, which in turn, reinforces conditioned fear responding. Thus, it is possible that VTA stimulation induced post-synaptic strengthening of the CS+US association on lateral amygdala neurons, which occurred regardless of whether the CS was explicitly paired with VTA stimulation. Therefore, it is likely that the VTA plays a regulatory role in the acquisition and/or relinquishment of learned fear by activating well-established fear

arousal pathways.

## 4.2 Effects of substantia nigra (SN) stimulation versus VTA stimulation

### 4.2.1 *Main finding*

The release of dopamine during fear arousal is not localized to the VTA and mesoamygdaloid pathway, but occurs in diverse areas of the brain (Kalivas, 1993). But, it would be erroneous to conclude that dopaminergic activation acts uniformly in distinct neural regions. Therefore, in this study, it was necessary to examine whether extinction retardation was specific to intra-VTA stimulation or extended to other dopaminergic mesencephalic regions.

The final experiment replicated the main experiment but used electrode implantation to deliver electrical stimulations of 500  $\mu$ A into the substantia nigra (SN) instead of the VTA. Stimulation delivery was paired with non-reinforced presentations of the light (CS). Normal extinction was observed in control animals and animals that received intra-SN stimulation during extinction training. This finding suggests that not all mesencephalic dopaminergic regions interfere with inhibitory fear processes. The difference in conditioned responding between the VTA-stimulation group and the SN-stimulation group was the most enlightening finding of this study. If, during normal extinction training there is a reduction in dopaminergic activity because the non-reinforced CS becomes more predictable (Collins and Pare, 2000), then these findings suggest that VTA-stimulation negated this inhibitory process, and this was not instrumented by SN stimulation.

#### 4.2.2 *Considering a functional dissociation between two mesencephalic dopamine systems*

This study provides further behavioural evidence for the functional dissociation between these two adjacent regions that support two anatomically distinct dopaminergic pathways: the mesolimbic and nigrostriatal systems (Ungerstedt, et al., 1974; Deutch et al., 1985). According to this view, dopamine neurons originate in the VTA and project to the ventral striatum, triggering reinforcement and motivational processes (Suri & Schultz, 1999; Wise, 2002). Whereas, successful extinction learning exhibited by rats that received SN stimulation conforms with the view that activation of dopamine neurons of the SN that project to the dorsal striatum contribute to the expression of motor responses and not emotional arousal (Hornykiewicz, 1979; Alexander and Crutcher, 1990; Dayan & Balleine, 2002; Alcaro, Huber, and Panksepp, 2007). This might explain why rats that received SN stimulation exhibited normal extinction learning.

A similar dissociation was declared when a past study demonstrated an increase in dihydroxyphenylacetic acid (DOPAC) levels within A10 (VTA) but not A9 (SN) dopamine cell bodies in response to footshock and conditioned stress (Deutch et al., 1985). Converging evidence from past studies indicates that elevations of dopamine levels within the mesolimbic system correspond with presentations of aversive stimuli (Thierry et al., 1976; LeMoal and Simon, 1991; Salamone, 1994; Berridge and Robinson, 1998; Schultz, 1998; DiChiara et al., 1999; Horvitz, 2000).

Results from the current study suggest that the SN may not be sensitive to fear-inducing stimuli. Electrical activation of SN neurons did not induce a stress response or evoke fear, despite the former association between the CS and footshock. It was also observed that intra-SN stimulated animals behaved more calmly following the procedure than intra-VTA stimulated animals. Another hypothetical explanation is that stimulation of the SN during extinction training improved attention by signalling each CS/light presented

without footshock. This might have reinforced the new extinction association, thus, over-riding the old conditioned association.

According to Bjorklund and Dunnett (2007), it is simplistic to assign VTA derived dopaminergic projections (A10) strictly to the amygdala and cortical regions, and nigrostriatal (A9) projections only to the striatum. In fact, A9 projections also innervate cortical and limbic areas, and A10 neurons also innervate the ventral striatum and the head of the caudate-putamen within the rat brain. Therefore, it is possible that stimulation of the SN facilitates extinction learning via ascending projections to the PFC (which has been identified as a dominant region of behavioural inhibition and in particular, fear extinction) (Morgan et al., 1993; Morrow et al., 1999; Quirk et al., 2000; Santini et al., 2001; Myers & Davis, 2002). Although a significant difference was not found between SN stimulated rats and controls following the extinction procedure, in comparison with all other extinction control groups, the SN group exhibited the smallest difference in startle amplitude in response to noise alone versus light + noise. This might suggest that SN stimulation did facilitate extinction learning, an effect that has also been shown with intra-mPFC stimulation during extinction sessions (Milad and Quirk, 2002).

Another important consideration is that only 3–5% of neurons in the SN are dopaminergic (Chinta and Anderson, 2005), whereas 70% of VTA neurons are dopaminergic (Swanson, 1982; Oades & Halliday, 1987). Although there is a possibility that non-dopaminergic neurons within the VTA were stimulated during extinction training, there is even greater probability that non-dopaminergic neurons of the SN were stimulated. Therefore, stimulation of non-dopaminergic SN neurons might explain why extinction retardation did not occur. For example, there is a histaminergic system that has been identified within the SN that is now recognised as an important neurotransmitter in synaptic plasticity and learning (Lui, et al., 2007). Presently, it is unclear what role, if any, this system plays in the acquisition of inhibitory associative learning. But, it is important that alternative systems are investigated before any conclusion is drawn regarding nigrostriatal dopaminergic involvement in

the extinction of conditioned fear, as the apparent dissociation between the two systems could be a function of competing non-dopaminergic systems.

One final consideration is that the stimulation electrodes may not have been implanted in the optimal region within the SN. This could be an important factor when considering why electrical stimulations of the SN did not impede extinction learning. Because the boundaries of midbrain dopamine regions are somewhat ambiguous (Oades & Halliday, 1987), it was intended that electrodes end centrally within the SN to ensure that the VTA was not accidentally stimulated.

There are two compartments within the substantia nigra: the pars compacta (SNc) and the pars reticulata (Winn, 2006). Upon site verification it was discovered that all electrodes were located within the substantia nigra pars reticulata (SNr), which could limit the implications made from this study. The SNc projects widely to the basal ganglia and the SNr is the recipient of corticostriatal output projecting predominantly to the thalamus, superior colliculus and reticular formation (Rosell and Amaya, 2000; Winn, 2006). The primary focus has been on the SNc in relation to the dopaminergic system and reward-based learning (Rosell and Amaya, 2000; Bjorklund and Dunnett, 2007). Moreover, electrophysiological data shows that cell responses from the VTA and the SNc do not significantly differ (Schultz, 1998). Thus, stimulation of the SNc instead of the SNr might have served as a better comparison with the results from the VTA/extinction experiment, particularly, if there is a reciprocal relationship between the nigrostriatal and mesolimbic pathways, which could interfere with the inhibition of learned fear.

However, it is a reasonable assumption that there was little difference between stimulating the SNc and the SNr because the two compartments are intimately related via deep dendritic connections (Bar-Gad, Morris, and Bergman, 2003). Concurrent activation of both the SNc and SNr was likely as the electrode probes used a span of approximately 2mm, which is large relative to the size of the midbrain in the rat. Therefore, it is very difficult to localize stimulation to one specific area. But, the clear difference between

VTA stimulation and SN stimulation on the acquisition of extinction learning, and the absence of motor or memory impairments across all stimulation groups (all rats had no difficulty remembering the Pavlovian association and displayed normal startle levels) suggests that a functional dissociation between the two dopaminergic regions exists.

#### 4.2.3 *Behavioural implications*

In light of the possible dissociation between the VTA and the SN, it can be speculated that activation of the VTA imposes a change in arousal levels that SN activation does not. For an animal to display enhanced fear-potentiated startle in response to a CS, a heightened state of arousal is necessary (Imperato, et al., 1992; Ikemoto and Panksepp, 1999). An animal's physiological status provides important internal information about whether an environment is safe or threatening and enables the animal to act appropriately (Damasio, 2003; Millan, 2003). And, an animal's mood or emotional state comprises the animal's internal context and contributes to the entire extinction learning complex (Bouton, et al., 2005; Baard, 2005). Yet, it is difficult for an animal to retrieve information learned while influenced by an emotional state (Bower, 1981; Eichenbaum, 1995). It is a reasonable assumption that VTA stimulation induced an anxious or aroused state (Imperato, et al., 1992; Kiyatkin, 1988; Horvitz, et al., 1997; Guarraci & Kapp, 1999; Pezze & Feldon, 2004; Millan, 2003), and made it difficult for animals to learn new, neutral inhibitory associations. Intra-VTA stimulated animals in the current study exhibited elevated cue-specific startle, suggesting heightened vigilance, which is an appropriate response to the expectation of impending footshock.

Therefore, VTA stimulation reinforced the fear-inducing context where the previous conditioned fear association occurred. In accordance with early behavioural theorists, Pavlov (1928), Skinner (1933), and Thorndike (1898), it could be suggested that VTA stimulation had a similar effect on behaviour as rewarding stimuli, in that stimulations 'stamped in' the relationship between the conditioned association and response habit. The key similarity between

stimulation used in the current experiment and reward is VTA activation, implying that VTA activation acts like excitatory stimuli and is likely to be behaviourally reinforcing. This underscores the point that excitatory stimuli can be either negative and fear-inducing, or positive and rewarding (Willick and Kokkinidis, 1995). Conversely, successful extinction exhibited by intra-SN stimulated animals indicates that SN activation does not induce a similar state of arousal, and does not reinforce the relationship between conditioned association and behavioural response.

Therefore, what is the role of dopamine neurons in the VTA versus the substantia nigra? It was noted by Pavlov (1927) that the presence of novel stimuli impairs extinction learning. A dominant argument has been that dopamine neurons respond only to sudden, novel and unpredictable stimuli (Redgrave et al., 1999), which could also encompass stimuli that was aversive or rewarding (Horvitz, 2000). On this basis, it was unlikely that the light (CS) would become a familiar and predictable stimulus because electrical activation of VTA dopamine neurons rendered the CS salient and unpredictable.

This raises the question of why electrical stimulation of either SN or VTA dopamine neurons did not have the same effect on behaviour if activation of all dopamine neurons signal a novel and/or salient stimulus. It is possible that all activated dopamine neurons signal a novel and/or salient stimulus, but that the behavioural response differs depending on the dopaminergic pathway activated. For example, a novel stimulus provokes arousal and behavioural excitement, which is mediated by dopamine neurons within the mesolimbic system, originating in the VTA (Ikemoto and Panksepp, 1999). Conversely, a novel stimulus might activate nigrostriatal dopaminergic neurons facilitating heightened attention and motor control, which is necessary for an animal to familiarize it with a new stimulus, and then learn an appropriate response. Mirenowicz and Schultz (1996) similarly proposed that different dopamine neurons determine positive versus negative motivational salience. Nevertheless, even if the two dopamine systems did activate different behavioural and cognitive responses (Deutch, et al., 1995), it is likely that under normal circumstances they would act simultaneously and be triggered



by the one stimulus, with each system modulating the function of the other. Thus, activation of the VTA could create a disinhibition of learned fear because SN activation might be required to suppress emotional responding.

The comparison between VTA results and SN results suggests that dopaminergic neurons play an integrative role in optimizing the shaping of behaviour and motor acts in relation to the demands of the environment (Grilner & Mercuri, 2002). Extinction deficits are an example of impaired adaptation because cessation of fear responding is expected when the environment changes from threatening to non-threatening. Based on the current findings, distortions of dopaminergic modulation within the VTA might contribute to impaired adaptation to the environment. However, it is important to take into account that the VTA and the SN were artificially and independently activated. Just as a single dopamine cell does not act in isolation, these two regions are integral parts of a very intricate neural network, which together, undoubtedly modulate a multitude of varying behaviours.

#### 4.3 Contributions, limitations, and future research

This study, conducted in the Psychology department at the University of Canterbury, contributes to the recent body of work examining the role of the mesolimbic system in the acquisition and relinquishment of learned fear in rats. In the late Professor Kokkinidis's laboratory, electrical or pharmacological manipulations were preferred to lesions of the mesolimbic regions as a means to model hyper-activation of neural fear circuitry. At the time, Professor Kokkinidis's researchers believed that impaired fear extinction learning were due to irrepressible activation of fear pathways in the brain. Disinhibition of learned fear has recently been identified as a potential factor causing fear-related, human psychopathologies (Fendt and Fanslow, 1999, Myers and Davis, 2004). Thus, the main contribution of the present study is the demonstration that extinction deficits arising from electrical stimulation of the VTA are similar to amygdaloid stimulation induced deficits (Kellett and

Kokkinidis, 2004).

#### *4.3.1 Issues surrounding stimulation specificity*

One main limitation of this study was an inability to isolate the specific response to VTA stimulation because the effect on adjacent regions and pathways cannot be ascertained. Even though possible unconditioned effects of VTA stimulation were largely eliminated, it remains difficult to ensure that 120 VTA stimulation of 500  $\mu$ A did not sensitise amygdaloid neurons. However, the final experiment for this study was critical because it demonstrated that electrical stimulation of the SN, which is immediately adjacent to the VTA, had no effect on extinction learning. Additionally, histological results indicated that stimulation to the supramammillary bodies (MM) had no effect on fear-potentiated startle, despite the MM being considered vital to the control of emotion including fear-conditioning (Millan, 2003). Therefore, animals stimulated in two regions closely situated with the VTA—the SN and the MM—which could have been indirectly activated during VTA stimulation, demonstrated normal extinguished fear.

Yet, it has been shown that the sensitising effects of VTA electrical stimulation on the amygdala do not always manifest behaviourally. For example, abnormal electrical activity of CE amygdaloid neurons (known as after-discharge) was evident following chronic, low current-high frequency intra-VTA stimulation (Gelowitz & Kokkinidis, 1999). One way to eliminate the possibility that VTA stimulation did not lower after-discharge thresholds of amygdaloid neurons would be to record electrical activity in the amygdala following 120 1-s electrical stimulations within the VTA. This would involve dual ipsilateral electrode implantation within the VTA and the amygdala. Electrical stimulation would be delivered to one bipolar electrode in the VTA and neural activity—recorded as an electroencephalographic tracing—would be measured from the other bipolar electrode. Animals would also have to undergo exactly the same experimental conditions as those reported in this study, and a comparative control group would be required.

#### *4.4.2 Considerations surrounding differences between VTA and SN stimulation*

Another important finding of this study was the discovery of a possible dissociation between the SN and the VTA within the fear extinction paradigm, particularly given the present controversy surrounding the action of dopamine cells and the dopaminergic pathways (Hornykiewicz, 1979; Wise, 1985; 2002; Puglisi Allegra et al., 1991; Koob & Le Moal 1997; Salamone et al., 1997; Schultz, 1998; Hollerman et al., 1998; Suri & Schultz, 1999; Redgrave et al., 1999; Guarraci & Kapp, 1999; Horvitz, 2000; Dayan & Balleine, 2002; Hyland et al., 2002; Robinson et al., 2005; Berridge, 2005; Gardner, 2005; Robinson et al., 2005). The fear-related properties of VTA and SN stimulation have been examined in this study, but previously, a majority of investigations have focused on the rewarding effects of VTA pharmacological and electrical activation. Even studies investigating the effects of electrical stimulation on induction of fear typically use high current levels delivered for longer than 1 second (Cuadra et al., 2000, Borowski, & Kokkinidis, 1996), making comparisons between this study and others, problematic. It is believed that the behavioural results observed in this study were not simply an artefact of electrical stimulation itself, because the experimental design aimed at reducing the likelihood of this possibility. In addition, it was shown that unlike the SN, VTA stimulation can also be fear provoking, and behavioural effects of VTA stimulation are likely to depend entirely on the context (signalled by either negative or positive stimuli) in which stimulations occur.

#### *4.4.3 Determining neurochemical effects*

Because the current study observed regional as opposed to neuronal activation, future research is warranted to examine the specific action of dopamine excitation in the VTA and the SN using similar experimental parameters. Dopamine plays a critical role in behavioural reinforcement and reward seeking behaviour, which in light of fear literature, suggests that mesolimbic dopaminergic activation is a response to both aversive and pleasurable stimuli and incites action (Wise, 2000; Schwarting and Huston,

1996). Although previous research conducted found that direct intra-VTA amphetamine infusion during extinction training interfered with extinction learning (Jackson and Kokkinidis, 2005, unpublished), experimental parameters differed and amphetamine effects on the SN were not explored. Psychomotor stimulants that promote dopamine transmission also reinforce excitatory stimulus- response patterns (Hill, 1970; Carr and White, 1984), and could enhance extinction learning when administered into the SN. Thus, further behavioural evidence confirming that dopamine neurons within the SN and the VTA function differently is an exciting possibility with considerable implications for future electrophysiological and cellular research.

#### *4.4.4 Addressing contextual influences*

Another limitation of this study was that the relationship between VTA stimulation and the experimental/testing context was not fully explored. Extinction deficits were consistent across both paired and randomized VTA stimulation/CS groups; therefore, it is asserted that electrical stimulation of the VTA reinforced the entire stimulus complex. However, it was not known whether startle levels would have remained elevated in response to the conditioned stimulus (light) if the testing context differed from the training context. Extinguished learned fear is relatively fragile and readily reinstated after a short sequence of footshock presentations, as long as animals are tested in the context where the footshocks were received (Bouton, 1984; Westbrook et al., 2002; Myers and Davis, 2004). This indicates that footshock is only part of a fear-provoking context that re-triggers conditioned fear. Although the current study demonstrated that VTA stimulation does not provoke unconditioned fear responses, it is not known whether VTA stimulation might reinstate conditioned fear when tested in the same training/testing chamber. Kellett and Kokkinidis (2004) found that electrical stimulation of the amygdala reinstated extinguished fear in the same training/testing context. A similar result would be predicted using VTA stimulation because the VTA and the amygdala appear to act in concert to generate fear arousal (Gelowitz and Kokkinidis, 1999).

A related issue is that VTA stimulation potentially induced an emotional state during extinction training, which was absent when fear-potentiated startle was tested 24 hours later. Bouton (1993) has pointed out that the learning context encompasses external surroundings and internal states of arousal, which must be consistent across both the training and testing contexts. Thus, an alternative interpretation of the results could be that extinction learning was not recalled on test day because the animals' internal states differed from those during which extinction learning was acquired. Therefore, the possibility that conditioned fear was extinguished could not be definitively ruled out, as the evidence only proves that conditioned fear responding continues when fear-potentiated startle is tested in the absence of VTA stimulation.

However, this limitation could be eliminated if fear-potentiated startle was tested within the extinction training session, and again 24 hours later. Myers and Davis (2002) observed the discrepancy between within session and retention of extinction learning. They cautioned against making generalizations about the involvement of a particular neural region in extinction learning based on one measurement of conditioned responding alone. Although a deficit that is evident 24 hours later can be presumed present during the extinction session, the current study was limited because VTA stimulation was induced during training but not during FPS testing. It is known that VTA stimulation alters an animal's emotional state (Rosen et al., 1996; Kalynchuk, 2000); therefore, it is problematic to claim that the training and testing contexts were consistent. In the future, the effects of VTA stimulation on extinction acquisition and retention need to be evaluated to control for context variability. In addition, it would be beneficial to study how long stimulated animals remained fearful of the light (CS) to examine whether VTA stimulation induced a long-term extinction deficit.

#### *4.4.5 Exploring effects of stimulation to alternative regions*

When this work was presented at the 23<sup>rd</sup> International Australasian Winter Conference on Brain Research (2005, Queenstown), it was suggested that the

effects of hippocampal lesions on extinction learning would be research-worthy if stimulation of the mesolimbic dopaminergic pathway does re-trigger learned fear memories. Although it is presumed that the hippocampus is involved in anxiety related behaviours (Gray, 1982; Millan, 2003), adding a lesion component to the current experimental paradigm would be complicating matters. Furthermore, hippocampal integrity is likely to be necessary for successful conditioning and extinction (Corcoran and Maren, 2001; Ji and Maren, 2005; Frohardt et al., 2000). However, there is some indication that the dorsal hippocampus supports context dependent aspects of extinction (Corcoran et al., 2005), and it would be interesting to examine whether hippocampal stimulation influences retrieval and/or reinstatement of extinction memories.

Stimulation of other midbrain regions would also be valuable. The periaqueductal grey (PAG) is an important site to consider because it is believed to govern the unconditioned fear circuit. This circuit mediates autonomic, reflexive and stereotyped behaviours that are not learned responses (Millan, 2003, Fendt & Fanselow, 1999). These defensive responses in animals are thought to be akin to symptoms of panic and stress disorders in humans (Adamec & Young, 2000; Parades, et al., 2000; Millan, 2003). Of relevance, dopamine fibres innervate the PAG, while non-dopamine efferents are sent from the VTA to the PAG (Oades & Halliday, 1987; Gifkins et al., 2002). Thus, these two structures are closely related, and unsurprisingly, similar effects appear to be found following electrical stimulation of either the PAG or the VTA (Di Scala et al., 1987; Borowski, & Kokkinidis, 1996). It is likely that PAG stimulation would induce unconditioned responses, which the current study demonstrated was not induced by VTA stimulation. Therefore, PAG stimulation paired with CS presentations during extinction training might serve as a suitable conditioned association, which might re-condition rather than extinguish animals. Nevertheless, this could serve as good comparative experiment for the current study.

#### *4.4.6 Exploring alternative measures of fearfulness*

Finally, it is acknowledged that there are numerous alternative experiments and measures that could be undertaken to enhance basic understanding of the neural and cognitive mechanisms driving fear extinction. For example, recording animals' hormonal levels following VTA stimulation would verify their state of fearfulness. Although the role of corticotrophin-release-hormone in fear-potentiated startle is unclear, it is important to consider whether stimulation of the VTA also increases endocrine responses to fear, which could evoke behavioural changes that might be readily re-conditioned during the extinction/stimulation procedure (Oades & Halliday, 1987; Walker et al, 2003). Freezing responses have also been used as a measure of extinction impairments (Nader and LeDoux, 1999), and it would be interesting to investigate how this defensive response is affected by VTA stimulation. Before substantive conclusions are made, this study should be replicated and ideally other measures of fear would be collated.

#### **4.5 General summary and concluding remarks**

In theory, it is probable that humans afflicted with anxiety and other fear-related psychopathologies have a hyper-responsive fear system. This could mean that their fear responses can be inappropriately enduring and disproportionate to the threat posed (Fendt and Fanslow, 1999). The recent view is that such disorders could reflect extinction learning deficits. An extinction deficit refers to an inability to extinguish a conditioned fear response to a non-reinforced stimulus that was once paired with an aversive event. The current study demonstrated that 120 sub-threshold, 1 second electrical stimulations of 500  $\mu$ A within the VTA during extinction training, interfered with rats' ability to extinguish conditioned responding to a conditioned stimulus (CS). It is of note that extinction deficits were consistent whether intra-VTA stimulation was immediately paired with a light presentation or delivered randomly. This suggests that VTA activation induced a generalized

enhancement of fear arousal, which directly reinforced the previously formed conditioned fear association. The possibility that VTA stimulation was aversive, or sensitized fear-potentiated startle responses was experimentally eliminated. It was concluded that suppressed activity of the mesolimbic pathway originating in the VTA is required for successful fear extinction learning. Results yielded by this study are remarkably similar to those reported by Kellett and Kokkinidis (2004) who found that electrical stimulation of the amygdala during extinction training also retarded extinction learning. These combined findings suggest that dysregulation of neuronal firing along mesolimbic pathways may be a probable cause of maladaptive fearfulness.

Currently, our knowledge of the neuroanatomical and neurobehavioral basis of Pavlovian conditioned fear has been dominated by the results of lesion studies, from which the absence of specific neural substrates infers their importance in the expression of fear. This could, however, be quite misleading. Given that fear straddles both behaviour and emotion, and fear associations are learned and remembered, it is likely that other regions beyond the amygdala are also critical to the acquisition and inhibition of conditioned fear. Moreover, this study has demonstrated that electrical stimulation enables isolated activation of a specific region from which a behavioural response can be observed.

Unlike extinction deficits induced by VTA stimulation, such deficits were not observed when rats received intra-substantia nigra stimulation, indicating that not all mid-brain regions have equal influence on inhibitory fear learning. There are conflicting views regarding the role of dopamine neurons, so behavioural evidence of a potential dissociation between mesolimbic and nigrostriatal dopaminergic pathways is particularly exciting. The preferred explanation for this dissociation is that dopamine neurons in both systems respond to arousing events—both rewarding and aversive—although each system motivates behaviour differently.

Understanding the neurobiological mechanisms underlying the inhibition of learned fear has only recently risen to prominence. Evidence from this and



past studies suggest that the VTA is not directly involved, but instead probably modulates the stress-response system that mediates behavioural expressions of fear. Nevertheless, the clinical implications for this study are two-fold. Firstly, it is possible that fear provoking memories and intrusive thoughts symptomatic of the psychological disorder, PTSD, could be triggered by emotionally arousing yet unrelated mild stressors in everyday life. Investigators commonly find increased dopamine levels in response to stressful or fear-provoking stimuli. Thus, activation of the mesolimbic dopamine system originating in the VTA could become a self-perpetuating cycle between mild stressors and traumatic memories, which ultimately reinforce and maintain maladaptive states of fear.

Secondly, extinction is the basic principle of behavioural therapies such as flooding used to treat fear-related psychologies that are cue specific. Emotional arousal induced by VTA stimulation was an important contextual component that impacted upon the animals, and ultimately impeded successful extinction learning. Successful treatment of conditions like PTSD or specific phobias requires consideration of contextual consistency between treatment context and everyday life, which includes an individual's mood and emotional states. The findings presented here could provide a neuroanatomical framework to posit possible triggers that maintain abnormal fearfulness.

The over-arching theme for this study has been to explore the paradoxical involvement of the VTA in both reward and fear driven behaviours. In animals and humans the VTA is particularly sensitive to the euphorigenic and fear eliciting properties of psychomotor stimulants and electrical stimulation. What has been learned from reward literature is that there is no specialized reward centre in the brain. And that those regions sub-serving reward circuitry may also be critically involved in normal and abnormal fear arousal due to the modulating role of VTA derived dopamine neurons. In the current study VTA stimulation was not rewarding but reinforcing—it reinforced conditioned fear responding to the light following extinction. Because intra-VTA stimulation is unsensed (Wise, 2002), its effect on emotionality depended implicitly on the

context in which stimulation was received. Thus, the paradox can be explained as interaction between context and emotional arousal, which can be rewarding in a context previously associated with a pleasurable experience, or fear provoking in a context previously associated with an aversive experience. Ultimately, both scenarios incite action that could also reinforce an association between contextual cues and behavioural outcome.

Finally, hyper-excitation of fear neurocircuitry has been implicated in acute shyness (Boyce & Ellis, 2005), paranoid psychosis related to drug addiction (Griffith, Cavanaugh, Held & Oates, 1968; Bell, 1973; Robinson & Becker, 1986; Sherer, 1988; Satel, Southwick, & Gawin, 1991; Yui, Goto, Ikemoto & Ishiguro, 1997; Borowski & Kokkinidis, 1998), schizophrenia (Russell et al., 2007), anxiety disorders (Rosen & Schulkin, 1998; Foa, et al., 1995; Shuhama et al., 2007) and inter-ictal behavioural disturbances related to epilepsy (Gloor, 1992; Kalynchuk, 2000). Understanding the neurobiological under-pinning of these disorders is paramount to successful treatment strategies. As an animal model, electrical stimulation of brain regions supporting fear neurocircuitry is a very useful method of investigating how hyper-excitation affects behaviour. This animal model also provides the opportunity to investigate potential suppressing agents. It is hoped that the current findings have helped to elucidate the role of the VTA in fear arousal, which might benefit future research.

## 5. References

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## 6. Appendix

### *Appendix A)*

#### **Substances that rats will self-administer directly into the VTA**

Systemically, rats learn quickly to self-administer amphetamine and cocaine, which up-regulates dopamine levels in the Acb and enhances rewarding MFB brain stimulation (Hoebel et al., 1983; Wise, 1998). Animals will also self-administer directly into the Acb the selective dopamine reuptake inhibitor: nomifensine (Carlezon et al., 1995), and dopamine itself (Dworkin et al., 1986). Morphine and delta opioids (Wise, 1998; Gardner, 2005; McBride, 1999), ethanol (Gatto et al., 1994), tetrahydro-cannabinol (THC) (Ikemoto, 2003; Ikemoto and Wise, 2004), nicotine, NMDA or AMPA (non-specific cholinergic agonists), carbachol (Ikemoto & Wise, 2002), and neurotensin are also readily self-administered directly into the VTA by animals. It is of note that the VTA is the only brain region that animals will directly self-administer ethanol (McBride, et al., 1999).